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(21) International Application Number: PCT/US96/15203 (22) International Filing Date: 20 September 1996 (20.09.96) (30) Priority Data: 60/004,023 20 September 1995 (20.09.95) US (71) Applicant (for all designated States except US): HEARTLAND BIOTECHNOLOGIES, L.L.C. [US/US]; 125 West 76th Street, Davenport, IA 52806-1340 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): DAS, Prem, O. [US/US]; Not furnished (**). MANDELL, Robert, B. [US/US]; Not furnished (**). BOULTON, Teri, G. [US/US]; Not furnished (**). McMULLEN, Thomas, W. [US/US]; Not furnished (**). (74) Agents: MURPHY, Gerald, M., Jr. et al.; Birch, Stewart, Kolasch & Birch, L.L.P., P.O. Box 747, Falls Church, VA 22040-0747 (US).		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: YEAST RECEPTOR AND G-PROTEIN FUSION PROTEIN (57) Abstract <p>The invention provides protein fusions between the C-terminus of heterotrimeric G-protein-coupled receptors and the N-terminus of either wild type or mutant G-alpha proteins of the yeast <i>Saccharomyces cerevisiae</i>. Methods are described for creating DNA constructs that encode such fusion protein, assays for correct expression of such fusion molecules in yeast, and assays for the coupling of such fusion molecules to the pheromone-induced signal transduction pathway of yeast. Furthermore, the invention encompasses yeasts expressing the fusion proteins and methods for screening compounds for activity as agonists or antagonists of seven-transmembrane receptor function.</p>		

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YEAST RECEPTOR AND G-PROTEIN FUSION PROTEIN

FIELD OF THE INVENTION

5 This invention covers protein fusions between the C-terminus of any G-protein coupled receptor and the N-terminus of the *Saccharomyces cerevisiae* G-alpha protein Gpalp, the DNA constructs encoding the same, yeast strains expressing the same, methods to ensure that the fusion protein is coupled to the yeast mating pathway,
10 and assays for such coupling.

BACKGROUND OF THE INVENTION

Papers of the scientific periodical and patent literature, and data archived in GENBANK by accession number, referred to herein throughout the text are
15 hereby incorporated in their entirety by reference.

Cell surface receptors recognize extracellular ligands such as hormones, nutrients and growth factors, and transduce the signal generated by ligand binding to effector molecules within the cell. An important class
20 of these receptors, variously called G-protein-coupled receptors, seven transmembrane domain receptors or serpentine receptors, is characterized by their interaction with heterotrimeric G-protein complexes comprised of alpha, beta and gamma subunits (Watson and
25 Arkinstall, *The G-Protein Linked Receptors Facts Book*, c. 1994 by Academic Press).

Activation of such receptors leads to dissociation of beta and gamma subunits from the alpha subunit, and consequent initiation of signaling cascades in the cell
30 by the dissociated components. Mammalian receptors of this class include the alpha- and beta-adrenergic, muscarinic cholinergic, cannabinoid, dopamine, opiate, serotonin, thrombin, platelet activating factor and thromboxane A2 receptors. Agonists and antagonists of
35 several of these receptors are important therapeutic agents, and many members of this class of receptors are involved in various disease processes. Therefore, there

is a need in the pharmaceutical industry for assays to identify new agonists and antagonists of these receptors from libraries of small molecules and peptides, for the purpose of new drug development.

5 However, the development of such assays has been hindered by several factors. The expression of many of these receptors is often limited to a specific cell type that is difficult to isolate or culture in quantity. Further, each receptor does not interact with all
10 members of the family of heterotrimeric G-proteins found in mammalian cells (which can include up to 20 alpha, 4 beta and 7 gamma isoforms), although some receptors interact with more than one G-alpha subunit. For many others, the cognate G-protein complex has not been
15 characterized. On the other hand, the same G-alpha protein or G-protein complex can interact with different receptors expressed on the same cell. Therefore, it is difficult to narrow down the physiologically important interaction in mammalian cell tissue culture. Ligands
20 for many of these receptors have been identified by binding assays using membrane preparations from tissue culture cells or heterologous systems such as insect cells overexpressing the relevant receptor. Ligands identified thus, however, may be agonists, antagonists
25 or neutral in terms of receptor function, since only binding and not activation is measured by these assays. Moreover, even binding assays cannot be used to study the so-called "orphan" receptors, which were identified by DNA homology methods, and whose physiological ligands and functions are unknown. Finally, these proteins
30 traverse the membrane seven times, giving rise to one free end and three loops on both sides of the membrane. Potentially, all three loops and the end could contribute to forming the ligand binding pocket on the outside and the recognition site for G-proteins on the
35 inside. These factors render it difficult to study these protein by X-ray crystallography and molecular

modeling. In addition, dividing these proteins into domains of specific function that can be analyzed separately, either by proteolysis or expression of gene fragments, is not feasible because of the loops. This
5 also renders this class of receptors less suited to rational drug design. Therefore, there is a need in the art for new and convenient assays to identify agonists and antagonists of these receptors.

The yeast *Saccharomyces cerevisiae* has already proven
10 useful in developing such assays. Two endogenous G-protein coupled receptors and one heterotrimeric G-protein complex have been characterized from this organism, all of which are involved in a developmental pathway leading to the formation of a diploid yeast cell
15 from fusion of two haploid cells of the a and alpha mating type. The two receptors, the a-factor receptor (encoded by *STE3*) and the alpha-factor receptor (encoded by *STE2*), are expressed respectively on haploid yeast cells of the alpha and a mating type, are activated
20 respectively by the a- or alpha- peptide factors secreted by cells of the opposite mating type, but trigger activation of the same heterotrimeric G-protein complex in both cell types. Activation of the complex releases the beta-gamma subunits, which activate the
25 mating pathway and cause expression of specific proteins that result in growth arrest at the G1 phase, and a morphological change from budded spheroidal cells to unbudded pear-shaped "shmoo" in preparation for mating. The genes involved in this signal transduction pathway
30 in yeast, how they interact to bring about G_i growth arrest in response to mating factor, and their similarity to mammalian signal transduction components (the thrombin pathway is chosen as an example) are represented in Fig. 1. (Jones, Pringle and Broach, *The Molecular and Cellular Biology of Yeast Saccharomyces*,
35 Vol. 2., c. 1992 by Cold Spring Harbor Laboratory Press).

Activation of heterotrimeric G-proteins requires a specific interaction between the receptor and the G-protein complex that is mediated primarily by the G-alpha subunit. Unactivated receptors are normally bound to a trimeric complex with inactive GDP-bound G-alpha. Receptor activation by the ligand stimulates GDP release from G-alpha followed by GTP binding and the dissociation of the beta and gamma subunits from the alpha subunit. In mammalian cells, this renders both G-alpha and G-beta-gamma "active" and capable of activating downstream signaling elements such as adenylyl cyclase. Hydrolysis of GTP to GDP switches G-alpha back to the inactive state, where it reassociates with G-beta-gamma to regenerate the inactive complex, which then associates with a receptor. In the yeast mating cascade, the entity that propagates the signalling cascade is the released complex of G-beta and G-gamma subunits; however, dissociation of that complex from G-alpha is still the crucial activation step.

Because G-alpha is the subunit that interacts primarily with the receptor, the affinity of a particular G-alpha for a given receptor largely determines which of the many heterotrimeric complexes in mammalian cells is associated with the receptor, and therefore determines the efficiency of coupling between a receptor and a given G-protein complex. (Conklin and Bourne, *Cell* 73:631 (1993)) Given this, the use of a heterologous systems such as yeast to model the activation of mammalian receptors is limited by the potential lack of interaction between yeast G-alpha and the mammalian receptor. For example, it has been shown the human beta-2-adrenergic receptor (BAR) can be expressed in *Saccharomyces cerevisiae* such that it is properly folded and located in the yeast plasma membrane, and binds extracellular ligands with affinities comparable to mammalian cells. However, ligand binding did not result in activation of the

5 mating response pathway, indicating that the mating cascade-associated G-protein complex comprising Gpalp, Ste4p, and Ste18p did not respond to BAR activation, possibly because of a lack of recognition between the yeast Gpalp and BAR. Activation was, however, achieved when the cognate human G-alpha protein was co-expressed (King et. al., *Science* 250:121-123 (1990)), indicating that the G-beta and G-gamma subunits of yeast could form a heterotrimer with the mammalian G-alpha protein that could respond to BAR activation by release of the beta-gamma complex.

10 These results indicate that co-expression of the cognate G-alpha subunit would be required to engineer a response of the yeast mating pathway to the activation of a heterologous receptor expressed in yeast. However, the physiologically relevant G-alpha-proteins have not been defined for many mammalian receptors, including the "orphan" types, limiting the applicability of this approach. Furthermore, these G-alpha proteins must bind to the yeast G-beta and G-gamma subunits so that no free beta-gamma complexes exist in the cell. They must be capable of responding to the ligand-binding signal by releasing the beta-gamma complex, and must undergo in the yeast cell the post-translational modifications that are needed for their function. All heterologous G-alpha proteins might not fulfill all of these criteria.

20 One way to potentially overcome these limitations is to adapt the endogenous yeast G-alpha protein such that it can be coupled to heterologous receptor activation. The invention described here is a means toward such adaptation of the yeast G-alpha protein. A critical and novel feature of our invention is the creation of a covalent linkage between a mammalian receptor and the endogenous yeast G-alpha protein, which is achieved by an in-frame gene fusion between the C-terminal end of the heterologous receptor gene and the N-terminal end of the yeast *GPA1* gene. The presence of the yeast G-alpha

protein as a linked moiety should greatly increase its local concentration and thus facilitate its interaction with the receptor and its response to activation of the receptor, as shown schematically in Fig. 2. Our invention also provides for the possibility that such facilitation is insufficient to overcome the lack of recognition between the two components. This is achieved by selection schemes used along with mutagenesis of the Gpalp domain of such fusion proteins, thereby identifying mutants in this domain in which activation of the receptor moiety is coupled to activation of the Gpalp moiety, and therefore to the yeast mating pathway.

Bertin et al (*PNAS* 91:8827-8831, 1994) have shown that protein fusions between the beta-2-adrenergic receptor (BAR) and its cognate mammalian G-protein, G_i-alpha, when expressed in mammalian cells, result in productive signal transduction as measured by ligand-dependent increase in cAMP levels. The cAMP response with the fusion was greater than in controls without the fusion, suggesting that covalent linkage enhances signaling efficiency. The authors suggest two reasons for the higher efficiency. One is that cycling between active and inactive forms of G-alpha may occur more rapidly in the chimera than in the unlinked state. The other is that the presence of the linked G-alpha may impede desensitization of the ligand response either by masking receptor determinants that mediate desensitization or by protecting G-alpha from degradation. However, unlike our invention, the article does not envision the use of the potentiated response to facilitate interactions between components that may not interact or only interact weakly, nor does it envision applications where the receptor and G-alpha protein are from different species.

Our use of receptor-Gpalp gene fusions in this manner is different from the method disclosed in the published

PCT application WO92/05244 for modeling G-protein coupled receptors in yeast. That method requires transformation of yeast with two exogenous genes, the receptor gene and the corresponding mammalian G-alpha protein gene, whereas our invention utilizes only the receptor gene, fusing it to a gene encoding a yeast G-alpha protein unlike the method disclosed in the WO92/05244 application, our invention is potentially applicable in cases where the G-alpha has either been not identified or does not interact with the yeast G-beta and G-gamma proteins. Published PCT applications WO 94/23025 discloses a method whereby the simultaneous expression of exogenous surrogates of yeast pheromone system proteins and modulators of these surrogates is used to identify peptide inhibitors or activators of the surrogate protein. However, those applications do not consider the use of a fusion protein, which is the basis of the present invention. Besides, the single fusion protein in our approach is not a surrogate of any individual yeast pheromone system protein but is simultaneously a surrogate of two distinct individual components. U.S. patent 5,030,576 covers the fusion of the ligand binding domain of a receptor to a reporter polypeptide that undergoes a conformational change upon ligand binding to the binding domain, but the application to G-protein-coupled receptors mentioned in the '576 patent describes the relevant reporter polypeptide as the cytoplasmic domain of such a receptor that is capable of interaction with G-proteins. Similarly, U.S. patent application WO 91/12273 covers hybrid proteins created by replacing domains other than the ligand-binding domain of a G-protein coupled receptor with corresponding domains of a yeast G-protein coupled receptor. In contrast to and as distinct from U.S. patent 5,030,576 and application WO/90 91/12273, our invention discloses a fusion between the full length mature receptor protein and not a fragment thereof with

defined properties such as the ligand binding domain and furthermore, uses the G-protein itself and not the cytoplasmic domain of another receptor as the N-terminus. In other words, we describe fusions between
5 two individual proteins from different species, in contrast to the approach commonly referred to in the literature as domain-swapping, where different domains with differing properties of a protein of similar structure from different species are fused together.

10 SUMMARY OF THE INVENTION

The present invention embodies the idea of using covalent linkage between two proteins created by gene fusion to potentiate their mutual interaction. The invention provides DNA constructs that encode and
15 express a fusion protein with a peptide bond between the C-terminus of any eukaryotic G-protein-coupled receptor and the N-terminus of the yeast G-alpha protein Gpalp.

The invention further provides yeast strains expressing these fusion proteins. The invention also
20 provides methods to ensure that these fusion proteins are synthesized and localized to the plasma membrane such that the Gpalp domain of the fusion protein can interact with yeast G-beta and G-gamma proteins. The invention further provides methods that can be used to
25 select, from a collection of mutants of the G-alpha domain of such fusion constructs, individual mutants demonstrating coupling of receptor activation to the mating pathway of yeast through the fusion protein. The invention further provides for use of the said strains
30 to identify small molecule agonists and antagonists of these receptors. The invention further provides for use of the said strains to identify peptide agonists and antagonists of receptor activation by transformation with a combinatorial peptide library, which is created
35 by expressing a randomized DNA sequence in yeast such that the individual peptides are secreted into the

medium via gene fusions to the signal peptide of the yeast alpha-factor.

DESCRIPTION OF DRAWINGS

5 Figure 1 shows G-protein signaling pathways in mammals and humans.

Figure 2 shows a map of plasmid pRMHBT4.

Figure 3 shows a map of plasmid pRMHBT10.

Figure 4 shows a map of plasmid pRMHBT18-NG.

Figure 5 shows a map of plasmid pRMHBT20-NG.

10 Figure 6 shows a map of plasmid pRMHBT26.

Figure 7 shows a map of plasmid pRMHBT41.

Figure 8 shows a map of plasmid pRMHBT43.

Figure 9 shows a map of plasmid pRMHBT44.

Figure 10 shows a map of plasmid pRMHBT45.

15 Figures 11A-11G show the nucleotide sequence encoding the STE2-GPA1 fusion protein and its amino acid sequence. The sequence starts at position 520 in STE2, and extends through position 1850 in GPA1. GenBank accession numbers for sequences are provided below. The
20 extra amino acids generated at the junction are underlined.

Figures 12A-12G show the nucleotide sequence encoding ThrR-GPA1 fusion protein and its amino acid sequence. The sequence starts at position 288 in ThrR, and extends
25 through position 1850 in GPA1. GenBank accession numbers for sequences are provided below. The extra amino acids generated at the junction are underlined. The STE2 leader sequence that proceeds and is linked in-frame to the ThrR sequence is also shown below.

DETAILED DESCRIPTION OF THE INVENTION

30 The following yeast strains are used in experiments constituting working examples disclosed in this application.

Table I: Strains Used in the Working Examples

<u>STRAINS</u>	<u>GENOTYPE</u>	<u>SOURCE</u>
MS16	mat a, <i>ade2-101</i> , <i>trp1D1</i>	Dr. M. Rose, Princeton Univ.
MS2288	mat a, <i>his3D200</i> , <i>leu2-3,112</i> , <i>trp1D1</i> , <i>ura3-52</i>	Dr. M. Rose, Princeton Univ.
HBS10	mat a, <i>ade2-101</i> , <i>far1-x200</i> , <i>his3D200</i> , <i>leu2-3,112</i> , <i>lys2DS738</i> , <i>trp1D1</i> , <i>ura3-52</i>	Heartland BioTechnologies
HBS10::pFFLZ	same as HBS10 except <i>far1::URA3-FUS1p-LACZ</i>	Heartland BioTechnologies
HBS32	mat a, <i>far1-x200</i> , <i>his3D200</i> , <i>leu2-3,112</i> , <i>trp1D1</i> , <i>ura3-52</i>	Heartland BioTechnologies
HBS12	mat a, <i>far1-x200</i> , <i>his3D200</i> , <i>leu2-3,112</i> , <i>ste2</i> , <i>trp1D1</i> , <i>ura3-52</i>	Heartland BioTechnologies
HBS12LZ	same as HBS12 except <i>leu2::LEU2-FUS1p-LACZ</i>	Heartland BioTechnologies
TMHY2-14A	mat a, <i>ade2-101</i> , <i>his3D200</i> , <i>lys2DS738</i> , <i>trp1D1</i> , <i>ura3-52</i>	Heartland BioTechnologies
TMHY2-223D	a/a, <i>ADE2/ade2</i> , <i>FAR1/far1</i> , <i>his3/his3</i> , <i>LEU2/leu2</i> , <i>lys2/lys2</i> , <i>trp1/trp1</i> , <i>ura3/ura3</i>	Heartland BioTechnologies
TMHY3D	a/a, <i>ADE2/ade2</i> , <i>FAR1/far1</i> , <i>GPA1/gpa1::TRP1</i> , <i>his3/his3</i> , <i>LEU2/leu2</i> , <i>lys2/lys2</i> , <i>trp1/trp1</i> , <i>ura3/ura3</i>	Heartland BioTechnologies
HBS14	same as TMHY3D	Heartland BioTechnologies
9A	mat a, <i>ade2-101</i> , <i>far1-x200</i> , <i>gpa1::TRP1</i> , <i>his3D200</i> , <i>leu2-3,112</i> , <i>lys2DS738</i> , <i>trp1D1</i> , <i>ura3-52</i>	Heartland BioTechnologies
9ALZ	same as 9A except <i>leu2::LEU2-FUS1p-LACZ</i>	Heartland BioTechnologies
9ALZAGS	same as 9ALZ except also <i>ste2</i>	Heartland BioTechnologies

Gene names are italicized (*GPA1*), and are in upper case (*GPA1*) when indicating a functional and dominant gene, and in lower case (*gpa1*) when indicating a non-functional recessive mutant gene. The corresponding proteins are in plain text (*Gpalp*). An agonist is defined as a molecule that binds to a receptor protein, and activates the receptor by inducing conformational or other changes in it such that the heterotrimeric G-protein complex that is bound to the receptor is disrupted, leading to release of the beta-gamma complex from the alpha subunit.

This invention embodies the idea of using covalent linkage between two proteins created by gene fusions to potentiate the interaction between G-protein-coupled receptors from other species and a protein homologous in function to the *Gpalp* protein of the yeast *Saccharomyces cerevisiae*. The experiments of Bertin et al have shown that there is a potentiation of the downstream response to receptor activation when the human beta-2-adrenergic receptor and its cognate G-alpha protein are linked in this manner. In addition to the two reasons considered by the authors which are described above, we consider that potentiation could also result from: a) more efficient coupling (which is considered in present models as transmission of a conformational change in the receptor to the G-protein complex) due to proximity of the interacting molecules brought about by covalent linkage; b) more efficient coupling because of the great increase in local concentration of *Gpalp* brought about by covalent linkage, thereby overcoming the effects of an unfavorable equilibrium binding constant for a heterologous receptor and yeast *Gpalp*; c) the presence of stoichiometric amounts of the two components, leaving no molar excess of either component to dilute the effects of ligand-mediated activation; d) better membrane anchoring of G-alpha by covalent attachment to the receptor compared to the normal situation of

anchoring via N-terminal myristoylation, which may be reversible. Regardless of the precise reason, it is likely that covalent coupling ameliorates the lack of recognition specificity between a given mammalian receptor and the yeast G-alpha protein.

The DNA constructions needed for the present invention can be made in vectors that can replicate independently in yeast cells, including the YCp or the YEp class of vectors or in vectors that are designed for integration into the yeast chromosome such as the YIp class. Most preferred vector are those which autonomously replicate in yeast.

G-protein-coupled receptors used in the present invention may be from animal species, including both vertebrates and invertebrates, plants or fungi other than *S. cerevisiae*. Preferred receptors are those from mammals, especially humans. Also, preferred are receptors from fungi, especially fungi that are pathogenic to humans. Mammalian receptors of this class that are encompassed by the present invention include, but are not limited to the following, whose nucleotide sequences are disclosed in the listed references:

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54. Substance K (neurokinin A) receptor: Gerard N. P. et al, J. Biol. Chem. 265: 20455 (1990).
- 30 55. Substance P (NK1) receptor: Gerard N. P. et al, Nature 349: 614 (1991).
56. Thrombin receptor: Vu T. K. et al, Cell 64:1057 (1991).
57. Thromboxane A2 receptor: Hirata M. et al, Nature 349:617 (1991).
- 35 58. Thyroid stimulating hormone (TSH) receptor:Nagayama Y. et al, Biochem. Biophys. Res.

Commun. 165:1184 (1989).

59. Vasoactive intestinal peptide receptor:
Sreedharan S. P. et al, Proc. Natl. Acad. Sci. USA
88:4986 (1991).

- 5 The present invention can also be practiced using any of the seven-transmembrane receptors encoded by nucleotide sequences presently deposited in GENBANK under the accession numbers listed in Table II:

Table II: List of Receptor Nucleotide Sequences

	Human acetylcholine m5 muscarinic receptor, 2261bp M80333
	Human acetylcholine muscarinic receptor, 2098bp M35128
5	Human activin type I receptor, 1518bp U14722
	Human activin type II receptor, 2382bp M93415
	Human adenosine receptor (A1) 2900bp L22214
	Human adenosine receptor (A1) brain hippocampus, 1267bp S45235
10	Human adenosine receptor (A2) 2383bp M97370
	Human adenosine receptor (A2), brain hippocampal, 2572bp S46950
	Human adenosine receptor (A2b) 1687bp M97759
	Human adenosine receptor (A3) 1739bp L22607
15	Human adenosine receptor (A3) 1767bp L20463
	Human adrenergic alpha 1a receptor 1860bp U03864
	Human adrenergic alpha 1a receptor 2002bp M76446
	Human adrenergic alpha 1a/d receptor 1831bp L31772
	Human adrenergic alpha 1b receptor 1560bp L31773
20	Human adrenergic alpha 1b receptor 1738bp U03865
	Human adrenergic alpha 1c receptor 1401bp L31774
	Human adrenergic alpha 1c receptor 1500bp U03866
	Human adrenergic alpha 1c receptor 1902bp U02569
	Human adrenergic alpha 1c receptor 2290bp D25235
25	Human adrenergic alpha 2 receptor gene 3604bp M23533
	Human adrenergic alpha 2 receptor kidney 1491bp J03853
	Human adrenergic alpha 2 receptor platelet 1521bp M18415
	Human adrenergic alpha 2c2 receptor 2072bp M34041
30	Human adrenergic alpha 2cII receptor 1382bp D13538
	Human adrenergic beta 1 receptor 1723bp J03019
	Human adrenergic beta 2 receptor 3451bp M15169
	Human adrenergic beta 2 receptor 3458bp J02960
	Human adrenergic beta 2 receptor, 2305bp Y00106
35	Human adrenergic beta 3 receptor, 1270bp M29932
	Human adrenergic beta receptor, brain, 1970bp X04827

- Human AH receptor 5,228bp L19872
- Human angiotensin II type 1 receptor 1575bp M93394
- Human angiotensin II type 1 receptor 2254bp M87290
- Human angiotensin II type 1b receptor 1563bp D13814
- 5 Human angiotensin II type 2 receptor (AGTR2) L34579
- Human angiotensin II type 2 receptor 5,293bp U20860
- Human angiotensin II type 2 receptor 1092bp U15592
- Human angiotensin II type 2 receptor 1439bp U16957
- Human angiotensin II type 2 receptor, 2476bp U10273
- 10 Human angiotensinogen II type-1A receptor 1829bp
M91464
- Human antidiuretic hormone receptor V2 (AVPR2) U04357
- Human arginine vasopressin receptor 1 (AVPR1) 6,402bp
U19906
- 15 Human arginine vasopressin receptor 1 (AVPR1) 1472bp
L25615
- Human arginine vasopressin receptor type II, U04357
- Human atrial natriuretic peptide clearance receptor
(ANP C- receptor) M59305
- 20 Human autocrine motility factor receptor (Ngp78)
1765bp L35233
- Human B-cell antigen receptor (MB-1) 681bp M74721
- Human bombesin receptor subtype-3, 1413bp L08893
- Human bradykinin B1 receptor 1082bp U12512
- 25 Human bradykinin B1 receptor, 4168bp U22346
- Human bradykinin BK-2 receptor, 1378bp M88714
- Human C5a anaphylatoxin receptor, 2328bp M62505
- Human calcitonin receptor 3588bp L00587
- Human calcitonin-like receptor, 2187bp U17473
- 30 Human cannabinoid receptor, 1755bp X54937
- Human cannabinoid receptor, central, long isoform,
2135bp X81120
- Human cannabinoid receptor, central, short isoform,
1252bp X81121
- 35 Human cannabinoid receptor, peripheral (CB2) 1790bp
X74328
- Human chemokine C-C receptor type 1 1495bp L09230

- Human cholecystokinin A receptor, 1393bp L13605
Human cholecystokinin A receptor, 1686bp L19315
Human cholecystokinin B/gastrin receptor brain, 1344bp
L08112
- 5 Human cholecystokinin receptor, 1969bp L04473
Human ciliary neurotrophic factor alpha receptor
L38025
Human ciliary neurotrophic factor receptor (CNTFR)
1566bp M73238
- 10 Human corticotropin releasing factor receptor, 1285bp
L23332
Human corticotropin releasing factor receptor, 1335bp
L23333
Human corticotropin releasing hormone receptor, 1146bp
15 U16273
Human CR2/CD21/C3d/Epstein-Barr virus receptor, 3934bp
M26004
Human CTLA4 counter-receptor (B7-2), 1112bp L25259
Human dopamine D1A receptor, 2337bp M85247
- 20 Human dopamine D2 receptor (DRD2), 2482bp M29066
Human dopamine D2 receptor, 1756bp M30625
Human dopamine D3 receptor (DRD3) gene, 1727bp U25441
Human dopamine D5 receptor (DRD5) gene, 1673bp M67439
Human EBV induced G-protein coupled receptor (EBI2)
25 1643bp L08177
Human EBV induced G-protein coupled receptor 2154bp
L08176
Human endothelial cell protein C/APC receptor (EPCR)
1284bp L35545
- 30 Human erythropoietin receptor, 1624bp M34986
Human erythropoietin receptor, 1818bp
Human Fc receptor low affinity CD16 (FcGR111), 1326bp
M24854
Human Fc-gamma receptor I A1, 1128bp L03418
- 35 Human Fc-gamma receptor I B1, 846bp L03419
Human Fc-gamma receptor I B2, 570bp L03420
Human Fc-gamma-R receptor leukocyte, 1977bp J04162

- Human Fc-gamma-receptor IIA (FCGR2A) M90727
Human Fc-gamma-receptor IIIB(FCGR3B) M90746
Human FMLP-related receptor II (FMLP R II) 1058bp
M76672
- 5 Human folate receptor 3 819bp U08471
Human follicle stimulating hormone receptor, 2186bp
M95489
Human follicle stimulating hormone receptor, 2393bp
M65085
- 10 Human formyl peptide receptor (FPR2), 1650bp M88107
Human formyl peptide receptor-like receptor (FPRL1)
2631bp M84562
Human G protein coupled-receptor (GPR12), 1230bp
U18548
- 15 Human G protein-coupled receptor (APJ) 1583bp
Human G protein-coupled receptor (EBI1), 2139bp L31581
Human G protein-coupled receptor (EBI1), 2215bp L31584
Human G protein-coupled receptor (GPR1) 1438bp L35539
Human G protein-coupled receptor (GPR1) 1438bp U13666
- 20 Human G protein-coupled receptor (GPR19) 2932bp U21051
Human G protein-coupled receptor (GPR3) 1262bp L32831
Human G protein-coupled receptor (GPR3) 3542bp U18550
Human G protein-coupled receptor (GPR4) 1365bp L36148
Human G protein-coupled receptor (GPR5) 1265bp L36149
- 25 Human G protein-coupled receptor (GPR6) 1477bp L36150
Human G protein-coupled receptor (GPR6) 2699bp U18549
Human G protein-coupled receptor (V28) 3100bp U20350
Human G-binding regulatory protein-coupled receptor,
M28269
- 30 Human galanin receptor, 1050bp U23854
Human galanin receptor, 1053bp L34339
Human gastrin receptor gene, 4754bp L10822
Human gastrin releasing peptide receptor (GRP-R)
1726bp M73481
- 35 Human glucagon receptor, 1578bp U03469
Human glucagon receptor, 2034bp L20316
Human glucagon-like peptide-1 receptor (GLP-1) 1567bp

L23503

- Human glucagon-like peptide-1 receptor, 1590bp U10037
Human glucagon-like peptide-1 receptor, 2431bp U01156
Human glucagon-like peptide-1 receptor, 2616bp U01104
5 Human glutamate receptor (GLUR5) 3188bp L19058
Human glutamate receptor (HBGR1) 2946bp M81886
Human glutamate receptor 2 (HBGR2) 3331bp L20814
Human glutamate receptor flip (GluR3-flip) 3056bp
U10301
10 Human glutamate receptor flop (GluR3-flop) 2747bp
U10302
Human glutamate receptor metabotropic subtype 5a,
4518bp D28538
Human glutamate receptor metabotropic subtype 5b,
15 4614bp D28539
Human gonadotropin releasing hormone receptor, 1541bp
L03380
Human gonadotropin releasing hormone receptor, 2160bp
L07949
20 Human growth hormone-releasing hormone receptor,
1617bp L01406
Human heat-stable enterotoxin receptor, 3745bp M73489
Human histamine H1 receptor, 1654bp D28481
Human histamine H2 receptor, 1191bp M64799
25 Human interleukin 8 low affinity receptor, 1510bp
M73969
Human interleukin 8 receptor alpha (IL8RA) 2007bp
L19591
Human interleukin 8 receptor B, 1750bp M94582
30 Human interleukin 8 receptor beta (IL8RB) 2856bp
L19593
Human interleukin 8 receptor type A (IL8RBA) gene,
4452bp U11870
Human interleukin 8 receptor, 1933bp M68932
35 Human leukemia virus receptor 1 (GLVR1), 3220bp L20859
Human leukemia virus receptor 2 (GLVR2), 3175bp L20852
Human luteinizing hormone-choriogonadotropin

- receptor, 2995bp M63108
Human lymph node homing receptor, 2354bp M25280
Human macrophage inflammatory protein-1-alpha/RANTES
receptor, L10918
- 5 Human major group rhinovirus receptor (HRV) 3003bp
M24283
Human mannose receptor, 5,185bp J05550
Human melanocortin 4 receptor, 999bp L08603
Human melanocortin 5 receptor (MC5R), 1262bp L27080
- 10 Human melanocortin 5 receptor gene, 1050bp U08353
Human melanocortin receptor, 1650bp Z25470
Human melatonin receptor, 1085bp U14108
Human monocyte chemoattractant protein 1 receptor
(MCP-1RA) U03882
- 15 Human monocyte chemoattractant protein 1 receptor
(MCP-1RB) U03905
Human N-formyl receptor-like 2 protein (FPRL2) 1198bp
L14061
Human N-formylpeptide receptor (fMLP-R26) 1281bp
M60627
- 20 Human N-formylpeptide receptor (fMLP-R98) 1866bp
M60626
Human N-formylpeptide receptor (FPR1) 6,931bp L10820
Human neurokinin 1 receptor (NK1R) 1230bp M76675
- 25 Human neurokinin 3 receptor (NK3R) 1755bp M89473
Human neurokinin A receptor (NK-2R) 1197bp M57414
Human neurokinin receptor (NK-1) 1466bp M81797
Human neuromedin B receptor (NMB-R) 1352bp M73482
Human neuropeptide Y peptide YY receptor, 1605bp
M88461
- 30 Human neuropeptide Y receptor (NPYR) 1225bp
Human neuropeptide Y receptor Y1 (NPYY1) 2881bp L07615
Human neuropeptide y receptor, 1470bp M84755
Human nucleotide receptor (P2U) 2030bp U07225
- 35 Human opiate delta receptor, 1136bp U10504
Human opiate mu receptor (MOR1) 2162bp L25119
Human opioid delta receptor, 1773bp U07882

- Human opioid kappa receptor (hKOR) 1154bp U17298
Human opioid kappa receptor (hKOR) 1182bp U11053
Human opioid kappa receptor (OPRK1) 1604bp L37362
Human opioid mu receptor variant (MOR1) 1473bp U12569
5 Human opioid receptor, 1610bp L29301
Human orphan G protein-coupled receptor, 1670bp L06797
Human orphan receptor (TR3) 2464bp L13740
Human orphan receptor (TR4) 2254bp L27586
Human oxytocin receptor, 3617bp X80282
10 Human oxytocin receptor, 4103bp X64878
Human PACAP receptor, 1664bp D17516
Human PACAP receptor, helodermin-preferring, 1640bp,
L36566
Human parathyroid hormone receptor, 1948bp L04308
15 Human parathyroid hormone/parathyroid hormone-related
peptide receptor, U17418
Human plasminogen activator receptor urokinase-type,
1608bp U08839
Human platelet activating factor receptor (PAFR)
20 1064bp M76674
Human platelet activating factor receptor (PTAFR)
1467bp M88177
Human platelet activating factor receptor, 1551bp
M80436
25 Human platelet-activating factor receptor, 1029bp
L07334
Human platelet-activating factor receptor, 1780bp
D10202
Human prolactin receptor (PRL) 2723bp M31661
30 Human prostacyclin receptor, 1979bp D25418
Human prostaglandin receptor (E2) 2052bp L25124
Human prostaglandin receptor (E2) 2372bp U19487
Human prostaglandin receptor (EP1) 1376bp L22647
Human prostaglandin receptor (EP2) 1958bp L28175
35 Human prostaglandin receptor (EP3) isoform IV, L32662
Human prostaglandin receptor (EP3A) 1729bp U13218
Human prostaglandin receptor (EP3A1) 1652bp U13216

24

- Human prostaglandin receptor (EP3D) 1540bp U13217
Human prostaglandin receptor (EP3E) 1429bp U13215
Human prostaglandin receptor (EP3F) 1456bp U13214
Human prostaglandin receptor (PGE-2), 1515bp L26976
5 Human prostanoid receptor EP3-I, 1870bp L27490
Human prostanoid receptor EP3-II, 1682bp L27488
Human prostanoid receptor EP3-III, 1379bp L27489
Human prostanoid receptor FP, 2494bp L24470
Human prostanoid receptor IP, 1417bp L29016
10 Human RMLP-related receptor I (RMLP RI) 1062bp M76673
Human RPE-retinal G protein coupled receptor (rgr)
694bp U15790
Human RPE-retinal G protein-coupled receptor (rgr)
1415bp U14910
15 Human secretin receptor precursor, 1650bp U20178
Human secretin receptor, 1616bp U13989
Human serotonin 1B receptor, (5-HT1B) 2635bp D10995
Human serotonin 1C receptor, 2733bp M81778
Human serotonin 1D receptor (5-HT1D) 1200bp M81589
20 Human serotonin 1D receptor (5-HT1D) 1260bp M81590
Human serotonin 1D receptor, 1348bp L09732
Human serotonin 1D receptor, 1506bp M89955
Human serotonin 1Db receptor (HTR1Db) 1959bp M75128
Human serotonin 1E receptor 5HTR1E, 1221bp M92826
25 Human serotonin 1E receptor, 1930bp M91467
Human serotonin 1F receptor (HTR1F) 1141bp L04962
Human serotonin receptor 5HT2 type 2 1368bp M86841
Human serotonin receptor 5HT7, 1406bp L21195
Human serotonin receptor, 1554bp L05597
30 Human serotonin receptor, 1938bp M83181
Human serotonin receptor, 2287bp M83180
Human soluble vascular endothelial cell growth factor
receptor (sflt) U01134
Human somatostatin receptor (SST) 1285bp L14865
35 Human somatostatin receptor (SSTR4) 1340bp L07833
Human somatostatin receptor isoform 1 (SSTR1), 1634bp
M81829

25

- Human somatostatin receptor isoform 2 (SSTR2) 1351bp
M81830
- Human somatostatin receptor subtype 3 (SSTR3) 1413bp
M96738
- 5 Human somatostatin receptor, 1427bp L14856
- Human substance P receptor (long form) 1674bp M84425
- Human substance P receptor (short form) 1268bp M84426
- Human thrombin receptor, 3472bp M62424
- Human thromboxane A2 receptor, U11271
- 10 Human thyroid hormone receptor alpha 1 (TR-alpha-1)
1876bp M24748
- Human thyroid stimulatory hormone receptor (TSHR)
2415bp M32215
- Human thyrotropin receptor (TSH) 2470bp M31774
- 15 Human thyrotropin-releasing hormone receptor, 1229bp
D16845
- Human transferrin receptor, 2826bp M11507
- Human vasoactive intestinal peptide receptor type 1
(V1RG) U11087
- 20 Human vasoactive intestinal peptide receptor, 2754bp
L13288
- Human vasoactive intestinal polypeptide receptor 2
(VIPR2) L40764
- Human vasopressin receptor (V2) 2282bp L22206
- 25 Human vasopressin receptor V3, 1869bp L37112

The protein analogous in function to the Gpalp of *Saccharomyces cerevisiae* can be, of course, the Gpalp of *S. cerevisiae*. In addition to the Gpalp protein of *S. cerevisiae*, there is also presently known the GPA2

30 gene of *S. cerevisiae* (Nakafuku et al., *Proc. Natl. Acad. Sci USA* 85:1374 (1988). The Gpa2p protein is not able to complement defective Gpalp function, but nevertheless the Gpa2p protein might interact with G-beta-gamma complexes to couple a seven-transmembrane

35 receptor to a biochemically selectable pathway. It is expected that other species of yeasts, for example

Schizosaccharomyces pombe, will also have proteins that can be used for the Gpalp protein in practicing the present invention.

5 In making the fusion construct, the seven-transmembrane protein is operatively linked to the protein having an activity analogous to the Gpalp of *Saccharomyces cerevisiae*. The two proteins can be directly fused; the carboxy-terminus of the seven-transmembrane protein being joined to the amino-terminus of the protein having Gpalp activity. 10 Alternatively, a short linker peptide can be used to join the two proteins. The linker is preferably from 1-25 amino acids long, more preferably from 1-20 amino acids long, still more preferably from 1-10 amino acids long and most preferably from 3-10 amino acids long. 15

In practice of one embodiment of the invention a "reporter" gene is operatively linked to the promoter of a gene analogous in function to the *FUS1* gene of *S. cerevisiae*. A reporter gene is one which signals the function of the expression cassette, typically of the promoter function, into which the reporter gene is inserted. The amount of the gene product of the reporter gene can be measured by immunoassay, by 25 enzyme activity (if the reporter gene encodes an enzyme) or by a metabolic selection strategy. Preferred reporter genes encode a protein that is not made by the yeast strain into which they are inserted, to avoid a high background result. Preferred reporter genes in implementing the present invention encode 30 enzymes whose activity can be measured colorimetrically or by a luminescence assay and include β -galactosidase, glucuronidase (GUS), green fluorescence protein, and luciferase. If a yeast strain in which the endogenous genes for them have 35 been knocked out is used, genes encoding alkaline phosphatase and invertase (SUC2) are also useful

reporter genes.

In a method for screening a compound for receptor antagonist activity, one contacts a yeast cell expressing a fusion protein comprising the seven-transmembrane protein of interest and a Gpalp that functionally couples to the mating-type pathway with the compound to be tested and with a ligand for said receptor. Then, the level of expression of a reporter gene, which measures the activity of a promoter that depends upon the activation of the mating-type pathway, for example, the FUS1 promoter, is measured. The level of reporter gene expression is compared in the presence and absence of the compound to be tested for antagonist activity. Antagonist activity is considered to be observed if the level of reporter gene expression, and thus activity of the mating-type activation-dependent promoter, is lower in the cell contacted with the compound being tested together with the ligand than in the cell contacted with the ligand, but not contacted with the compound being tested. By "lower" is meant a degree of difference between the reporter gene expression in the cells treated with the test compound together with ligand of at least 1/3. The larger the degree of difference, the greater the antagonist activity. A range of differences between 1/3 and 1/10 is expected. Preferably the range is 1/5 to 1/25. More preferably, the range is 1/5 to 1/50. Most preferably, the range is 1/50 to 1/200.

A method for testing a compound for receptor agonist activity is similar to the test for receptor antagonist activity. One contacts a yeast cell expressing a fusion protein comprising the seven-transmembrane protein of interest and a Gpalp that functionally couples to the mating-type pathway with the compound to be tested. Then, the level of expression of a reporter gene, which measures the activity of a promoter that depends upon the

activation of the mating-type pathway, for example, the FUS1 promoter, is measured. The level of reporter gene expression is compared in the presence and absence of the compound to be tested for agonist activity. Agonist activity is considered to be observed if the level of reporter gene expression, and thus activity of the mating-type activation-dependent promoter, is higher in the cell contacted with the compound being tested than in the cell not contacted with the compound being tested. By "higher" is meant a degree of difference between the reporter gene expression in the cells treated with the test compound together with ligand of at least 3-fold. Greater degrees of difference are preferred. An expected range is from 3 to 10-fold higher. An acceptable range is 3 to 8-fold higher. Preferably, the degree of difference is 10 to 25-fold. More preferably, the degree of difference is 20 to 100-fold.

A plasmid construct is made that expresses the Receptor-Gpalp fusion protein, and this plasmid is transformed into diploid yeast cells having one mutant and one wild type copy of the essential yeast G-alpha protein gene *GPA1*. Sporulation of the diploid should give two viable and two non-viable spores because *GPA1* is essential for haploid growth, unless the fusion protein contains a functional Gpalp domain. If so, more than two viable segregants will be obtained, providing a simple genetic complementation assay for appropriate expression and activity of the Gpal domain of the fusion protein. Next, assays based on mating pathway activation are performed, using known activators of the receptor domain of the fusion protein, to test whether the receptor domain of the fusion protein is functional and capable of transmitting the ligand-binding signal to the fused Gpal domain. If so, the fusion molecule is fully functional in both of its domains. If not, the same

assays can be adapted, in conjunction with mutagenesis of the Gpal domain, to select for mutants in which the intact receptor domains can signal to the mutant Gpal domain to activate the mating pathway upon activator
5 binding. A detailed description of the procedure is given below.

Yeasts can be tranformed with vectors encoding the recombinant DNA molecules of the present invention by means well-known in the art. Similarly, membranes
10 from yeasts expressing the recombinant DNA molecules of the present invention can be prepared and stored by methods well-known in the art.

Step 1: engineering a covalent linkage between the full length receptor (excluding the cleaved signal peptide, for reasons given in step 2) and Gpalp at their respective carboxy and amino terminal ends.
This is achieved by fusing the genes in frame by standard methods of molecular biology (Maniatis, Fritsch and Sambrook, *Molecular Cloning, a Laboratory
20 Manual*, 2nd Ed. c. 1989 by Cold Spring Harbor Laboratory Press.), as illustrated in examples 1 and 2. The fusion construct includes in addition the endogenous 3' processing signals of the GPA1 gene for proper termination of transcription and
25 polyadenylation. The construct can be made in a vector that can either replicate autonomously in yeast cells, or that integrates into the yeast chromosome. The vector additionally includes a transformation marker gene so that the final construct can be
30 transformed into yeast cells and transformant selected by using the marker.

Step 2: engineering the fusion protein for yeast plasma membrane expression. This is achieved by replacing part of the signal sequence of the receptor
35 in question with part of the N-terminal signal

sequence of the yeast G-protein-coupled receptor Ste2p. Any other N-terminal signal sequence that directs co-translational insertion across the rough endoplasmic reticulum membrane may also be used; examples include N-terminal signal sequences of the a-factor receptor *STE3* or secreted proteins such as invertase, and alpha mating factor precursors *MFa1* and *MFa2*. Attachment of the signal sequence is done by an in-frame fusion of a DNA fragment encoding the signal peptide, preferably from Ste2, with the DNA fragment encoding the construct from step 1 by standard methods of molecular biology, as illustrated in example 2. Similar constructs have been shown to cause yeast plasma membrane expression of the human beta-adrenergic receptor (King et al., *Science* **250**:121 (1990)) and the muscarinic cholinergic m5 receptor (Huang et al., *Biochem. Biophys. Res. Comm.* **182**:1180 (1992)) with ligand binding characteristics that closely mimic the native receptor in mammalian cells.

Step 3: placing the construct under the control of a yeast promoter. This is achieved by cloning in an appropriate promoter fragment contiguous to the 5' of the construct. Preferred promoters are those which can replicate autonomously in yeast. Example 1 demonstrates how this can be done using inducible and moderately strong *GAL* promoter. Example 3 describes constructions using the strong and constitutive *PGK* promoter. Codon usage in yeast is biased such that genes expressed at high levels use only one or two of the several possible degenerate codons to encode amino acids. (Jones, Pringle and Broach, *The Molecular and Cellular Biology of Yeast Saccharomyces*, Vol. 2, c. 1992 by Cold Spring Harbor Laboratory Press.) A strong promoter such as the *PGK* promoter may therefore be required to generate sufficient RNA levels to overcome the lack of codons preferred by yeast in

receptor genes from other species. Alternatively, the receptor-encoding DNA can be engineered to utilize preferred yeast codons.

Step 4: mutating the FAR1 gene. Far1p is required for growth arrest induced by activation of the mating pathway. For the assays described in Steps 10 and 11, the far1 mutation is needed to enable haploid cells with an activated mating pathway to grow while retaining other features of mating pathway activation. The FAR1 gene can be mutated by replacement with another auxotrophic marker gene (Scherer and Davis, *Proc. Natl. Acad. Sci. USA* 76:4951), or by the two-step mutation strategy (Rothstein, *Methods in Enzymology*, 101:202). The latter method is described in Example 11.

Step 5: constructing diploid yeast cells with one wild type and one mutant copy of GPA1. Because GPA1 is an essential gene for haploid cell growth and cannot be mutated in haploid cells directly, the mutation has to be made in a diploid strain preferably a mutant strain having several auxotrophic marker genes on both copies of its chromosomes. Diploid cells of this genotype are constructed by disruption of one of the two GPA1 copies by integration of an auxotrophic marker gene, as in example 5 where the TRP1 gene is used. In subsequent segregation, the mutant copy can be followed by the TRP1 marker. Thus, because GPA1 is essential, sporulation of each tetrad should give two large colonies, and two small or undetectable colonies, and both of the large colonies should require tryptophan for growth, i.e. lack the TRP1 gene. This is illustrated in example 5.

Step 6: transforming the construct of Step 3 into the diploid strain of Step 5. The construct of Step 3 is

cloned into a yeast vector that can replicate as a plasmid, and carries a gene that complements one of the auxotrophic mutations present in the diploid strain used to create *gpa1* and *far1* mutations in Steps 4 and 5. Replicating vectors based on either a yeast centromere sequence, exemplified by the YCp series of vectors, or the 2-micron plasmid origin, exemplified by the YEp series of vectors can be used. (Rose and Broach, *Methods in Enzymol.* 194:195.) The plasmid is then cloned into the diploid strain of Step 5, and transformants carrying the plasmid are selected on the basis of a marker present in the plasmid, preferably an auxotrophic marker, which is *URA3* in the case of YEp and YCp vectors.

Step 7: genetic complementation method for testing function of Gpa1 domain of the receptor-Gpa1 fusion. Sporulation of the diploid strain of step 6 carrying the fusion construct provides a convenient way to test if the Gpalp domain in the fusion construct can functionally replace the Gpalp gene product. Segregation of *GPA1* and *FAR1* in the diploid strain from Step 5, of genotype *GPA1/gpa1; FAR1/far1*, should yield the following four haploid genotypes: (i) *GPA1;FAR1* (ii) *GPA1;far1* (iii) *gpa1;FAR1* and (iv) *gpa1/far1*. Haploids with genotypes i and ii should give viable colonies, those with genotype iii should not give a detectable colony and those with genotype iv should give very small colonies because of incompleteness of growth arrest due to *far1*. If random spores from this population are analyzed, each of these genotypes should occur at equal frequency. However, because of independent assortment in each tetrad, the two spores that carry *gpa1* from a single meiotic event may be both *FAR1*, both *far1*, or one of each. Therefore, dissection of any tetrad should always yield two large colonies, and two others which

may be both very small (genotype *gpa1; far1*), both invisible (genotype *gpa1, FAR1*) or one of each. Such segregation is illustrated in examples 5, 6, 7 and 8, where tryptophan prototrophy is used to follow segregation of *gpa1::TRP1*, and a PCR assay is used to follow segregation of *FAR1*.

If the initial diploid cell carried a plasmid, it should be present in all four spores of the tetrad with equal probability. This probability is always less than one since plasmids can be lost at some frequency in the mitotic divisions preceding meiosis where selection for the marker carried on the plasmid is relaxed, and also in the two divisions of meiosis. If this plasmid carried a gene capable of fully complementing the *gpa1* mutation, then dissection of each tetrad would yield two large colonies as before due to the presence of *GPA1*, and of the two remaining spores of genotype *gpa1*, some would yield large colonies due to complementation. Thus, some tetrads would show 3:1 or 4:0 segregation for large vs. small or invisible colonies, and the presence of segregants of this type is indicative of complementation. This is illustrated in example 6a, for the *GPA1* gene expressed from its own promoter, example 6b for the *GPA1* gene expressed from the *PGK1* promoter, example 7 for a *STE2-GPA1* in-frame fusion protein expressed from the *PGK1* promoter, and example 8 for an in-frame fusion protein between the thrombin receptor and *GPA1* expressed from the *PGK1* promoter.

Step 8: confirmation of the functionality of the Gpa1 domain of fusion proteins. Step 7 describes how simple segregation analysis of genetic complementation can provide a good indication of the function of the Gpa1 domain. However, other genetic phenomena can also give rise to deviations from 2:2 segregation. For example, gene conversion of the

disrupted *gpa1* by the wild type copy, either in meiosis or in the mitotic divisions preceding mitosis could give rise to 3:1 or 4:0 segregation respectively. Theoretically, gene conversion could also occur between the complete coding sequence of GPA1 present on the plasmid construct and the disrupted chromosomal copy. To eliminate these possibilities, the presence of two chromosomal *gpa1* mutants in each tetrad is identified by segregation of the auxotrophic marker gene whose insertion was the means of disrupting, and thus mutating, one copy of GPA1 in the diploid strain in Step 4. Gene conversion of types described above restoring a complete GPA1 gene should lead to loss of this marker, and thus to the presence of less than two haploid spores carrying this marker in each tetrad.

In addition to the above possibility, all diploid cells that sporulate might not carry the plasmid since it is lost at some frequency in mitosis unless selection for the plasmid is maintained. Diploid cells can undergo several mitotic divisions without selection prior to meiosis in the sporulation medium, which may lead to loss of the plasmid and thus give rise to 2:2 segregation. In the analysis of Step 7, this would be incorrectly interpreted as an inability of the plasmid to complement *gpa1*.

To eliminate the above possibilities, the four colonies from each tetrad are tested for growth on media that detects the presence of the marker that disrupts the GPA1 gene (*TRP1* in example 5), and the plasmid marker (*URA3* in examples 6, 7 and 8). In the event that there is no complementation and no plasmid loss, 2:2 segregation should be seen in each tetrad, both large colonies should be *trp*⁻, any very small colonies (carrying *far1*) should both be *TRP*⁺ and a variable number of both large and small colonies should carry the plasmid and therefore be *URA*⁺. If

there is complementation with no plasmid loss, all segregants from each tetrad should form large colonies, two of which are *trp⁻* and two *TRP⁺*, and all should be *URA⁺*. In the more likely possibility of complementation with some plasmid loss both in mitosis and in meiosis, tetrads would segregate 2:2 (plasmid loss in mitosis), 3:1 (plasmid loss in meiosis) or 4:0 (no plasmid loss). In 2:2 segregants, both large colonies would be *trp⁻*, and none would be *URA⁺*. In 3:1 segregants, two colonies would be *trp⁻* and variably *URA⁺*, and one *TRP⁺* colony would always be *URA⁺*. In 4:0 segregants, two *trp⁻* colonies would be variably *URA⁺*, and two *TRP⁺* colonies would always be *URA⁺*. Data illustrating such analysis are provided in examples 6, 7 and 8.

Step 9: tests for function of the receptor domain. Binding assays provide a sensitive assay for proper expression of the receptor fusion protein, its targeting to the yeast plasma membrane and appropriate folding and generation of transmembrane domains to generate the extracellular binding site. Scatchard analysis of binding data can provide measurements of binding affinity, which can be compared to the affinity in mammalian cells expressing wild-type receptor to obtain a further measure of appropriate expression. Scatchard analysis also provide measurements of the number of binding sites for ligand per cell, which is a good measure of expression levels.

In the examples cited here, however, we have used the more stringent alternate approach described in step 10, which not only requires binding to the receptor domain of the fusion protein, but also requires transmission of the binding signal through the linked *Gpa1* domain to the mating factor pathway.

Step 10: mating and shmoo formation assay for coupling of receptor domain activation to mating pathway activation. Activation of the mating pathway in haploid cells leads to a distinct morphological change from the typical ovoid cells of vegetatively growing yeast to a pear-shaped "shmoo" which enables mating with cells of the opposite mating type if they are present. In examples 10 and 12 describing a protein fusion between the yeast receptor Ste2p and Gpalp, we have used both the shmoo formation assay and the mating assay to detect functional coupling between the covalently linked domains. The mating assay can only be used with the endogenous yeast receptors Ste2p and Ste3p, because this requires a response to mating pheromones secreted by another yeast cell of the opposite mating type, but the shmoo formation assay can be adapted to other receptors from heterologous organisms.

Step 11: beta-galactosidase induction assay for coupling of receptor domain activation to mating pathway activation. This method uses a mating pathway-inducible promoter operatively linked to the bacterial beta-galactosidase gene (*lacZ*) as a reporter. For example, transcription from the FUS1 promoter is stimulated by activation of the mating pathway, and therefore, in cells carrying FUS1-*lacZ* constructs, induction of beta-galactosidase becomes a sensitive indicator for receptor activation. Examples 9b, 10d, 12, 13 and 14 describe this assay for wild type cells to characterize the method (9b), Ste2p-Gpalp protein fusions (10d, 12) and thrombin receptor-Gpalp fusions (13, 14). Because expression of beta-galactosidase is easily quantitated by spectrophotometry, a quantitative measure of coupling is obtained by means of this assay.

The FUS1-beta-galactosidase construct can be

transformed into the haploid strain from Step 8 and maintained on a replicating plasmid of the YEP type. This gives higher basal values of β -galactoridase due to the 50-100 copies of the plasmid present in each cell, as shown in example 9b. Alternately, the basal expression level can be reduced by integration of the construct into the chromosome, as shown in examples 9b, 10d, 12, 13, and 14 for integration into the FAR1 locus and the LEU2 locus.

10 **Step 12: growth assay for coupling of receptor domain activation to mating pathway activation.** In this case, a mating pathway-inducible promoter such as FUS1 is operatively linked to a an auxotrophic marker gene that is mutated in the cells to be tested. As in Step 15 10, activation of the mating pathway leads to expression of the auxotrophic marker gene, conferring the ability to grow in appropriate media that lacks the final end product of the marker enzyme. We have used the LYS2 gene in this manner in example 10c. The 20 particular advantage of LYS2 (and also URA3) is that expression of this gene can be selected for in lysine deficient media as well as selected against in media containing the reagent alpha-aminoadipate. This renders the assay adaptable to screening for both 25 agonists and antagonists of the receptor that is modeled. The use of a FUS3-LYS2 construct to assay agonists is illustrated in example 9a and 10c involving activation of the mating pathway by a Ste2-Gpal fusion protein.

30 **Step 13: mutagenesis of the Gpal domain to increase coupling efficiency.** In the event that the results from steps 9, 10, and 11 do not indicate optimal coupling between receptor activation and the mating pathway in a given protein fusion, the G-alpha domain 35 of the fusion can be mutagenized by the standard

methods, including those described below, and mutants which are created thereby that confer increased coupling efficiency can be selected using the methods described in steps 10 and 11. Mutagenesis can preferably be effected using one or a combination of the following methods:

a) random mutagenesis by PCR amplification (Cadwell and Joyce, *PCR and Its Applications*, c. 1994 by Cold Spring Harbor Laboratory Press, esp. pp. S136) using primers homologous to the two ends of GPA1, with an Mlu I site in the 5' primer and a Pfl MI site in the 3' primer. In this method, amplification is performed in the presence of manganese and altered levels of magnesium such that a mutation rate of 0.5-1% per base is obtained. Products from the mutagenic amplification reaction will be cloned into the plasmid from step 3 which has been digested with MluI and Pfl MI enzymes, and additionally with Sph I to destroy the original GPA1 gene. The ligation mix will be transformed into *E. coli* such that a library of $>10^6$ clones is obtained, representing that many individual mutations. Plasmid DNA from a bulk plate growth of the entire transformation mix will be used to transform yeast and select for mutants with functional coupling as described by the selection procedure of step 11 or the screening procedure of step 10.

b) site-directed mutagenesis of specific regions of GPA1 using a mixed degenerate oligonucleotide population synthesized with a central region with degenerate bases that targets the domain to be mutagenized, flanked by 5' and 3' regions that are fully homologous to the GPA1 gene. Following standard methods of oligonucleotide mutagenesis, the primer extension mixture will be transformed into *E. coli* such that sufficient individual transformants are recovered to ensure adequate representation of the pool of mutants. The entire library of mutants will

then be recovered from bulk growths and used as in a above. Regions to be mutagenized would include the carboxy terminal, which has been implicated in binding to the receptor, and other regions of weak homology.

- 5 c) loop-out mutagenesis using oligonucleotides with homology to regions that flank the region to be deleted. Comparison of the amino acid sequence of GPA1 to human Gs-alpha shows that several large regions of the GPA1 sequence are non-homologous to the human protein, and would be good candidates for loop-out mutagenesis (e.g. amino acids 1-61, 75-110, 142-188, 217-237 of the GPA1 sequence.
- 10

15 **EXAMPLE 1: CONSTRUCTION OF A FUSION BETWEEN THE YEAST ALPHA FACTOR RECEPTOR Ste2p AND G-ALPHA PROTEIN Gpalp UNDER TRANSCRIPTIONAL CONTROL OF THE GAL1 PROMOTOR**

a) Ligating the GAL1 promoter into the yeast vector

- YCp50: the yeast vector YCp50 was digested with BamHI and EcoRI, and the resulting 7572 bp fragment was purified from an agarose gel using GeneClean™ (Bio
- 20 101). An 806 bp EcoRI-Bam HI fragment carrying the Gal1 promoter (position #1-810 of GenBank accession number K02115, where a BamHI site was added to the 3' end) was ligated into this YCp50 fragment and the resulting plasmid is designated pRMHBT1.

- 25 b) Inserting a polylinker into pRMHBT1: the plasmid pRMHBT1 was digested with BamHI and PflMI and the resulting 7574 bp fragment was purified as in Example 1a. For annealing of the two oligos "a" and "b" listed below, a solution containing 20mM tris-HCl pH 7.4,
- 30 10mM MgCl, 50mM NaCl, and 400mM of each oligo were

heated to 70°C for 10 minutes, and cooled slowly to 25°C (15 minutes).

oligo a) 5' GATCCGCGGCCGCGACGCGTCCAGCCC'

oligo b) 5'CTGGCAGCGTGCGGCCGCG'

5 These oligos anneal to form a polylinker with BamHI, NotI, MluI and PflMI sites, in that order. The annealed oligo fragment was then cloned into the 7574 pRMHBT1 BamHI/PflMI fragment to make pRMHBT2.

c) Ligating GPA1 into pRMHBT2: The plasmid pRMHBT2 was
10 cut with MluI and PflMI, and the 7585 bp fragment was purified as above. GPA1 was amplified by PCR from a *Saccharomyces cerevisiae* genomic DNA prep using the following two primers:

oligo c) 5'GACACGCGTGTAATGGGGGTGTACAGTGAGTACGC'

15 oligo d) 5'CGTCCAAGGGATGGACCTTTTTTTTCTCATGCG'

Bold text represents the GPA1 sequences and normal text represents additional nucleotides (this convention will be maintained throughout this text). Oligo "c" contains bp 200 to 223 of the GPA1 sequence
20 (GenBank accession number M15867) and 10 additional nucleotides containing a MluI restriction site. Oligo "d" contains bases complementary to residues 1829 to 1850 of the GPA1 sequence and additional nucleotides creating a PflMI site homologous to the PflMI site at
25 position 1610 in YCp50. PCR amplification of yeast genomic DNA with these oligos yields a GPA1 fragment that contains nucleotides 200-1850 of the GPA1

sequence. The MluI site is immediately upstream of the ATG start codon, and the PflMI site is 232 bp downstream of the TGA stop codon. The amplified GPA1 fragment was digested with MluI and PflMI and ligated to the 7583 bp MluI/PflMI fragment of pRMHBT2 to make pRMHBT3.

d) Ligating STE2 into pRMHBT3 as an in-frame fusion to GPA1: STE2 was amplified by PCR from *Saccharomyces cerevisiae* genomic DNA using the following 2 primers:

10 oligo e) 5'CGGGATCCAAGAATCAAAAATGTCTGATG3'

oligo f) 5'GAACGCGTTAAATTATTATTATCTTCAGTCC3'

Oligo "e" contains nucleotides 520 to 544 of the STE2 sequence (GenBank accession number M24335) and 4 additional nucleotides which create a BamHI restriction site. Oligo "f" contains bases complementary to nucleotides 1804 to 1827 of the STE2 sequence and eight additional nucleotides which include a MluI site. PCR amplification yields a STE2 fragment containing nucleotides 520-1827 of the STE2 sequence, and includes the entire coding sequence from the ATG start codon (pos. 535, underlined in the oligonucleotide sequence "e" above) to the last base of the Ste2p C-terminal leucine codon (pos. 1827, underlined in the oligonucleotide sequence "f" above). The STE2 PCR product was cut with BamHI and MluI and ligated to the 9224 bp BamHI/MluI fragment of pRMHBT3 to make pRMHBT4. The MluI junction forms an in-frame

fusion between *STE2* and *GPA1*; the resulting chimera codes for all of Ste2p, a tripeptide thr-arg-val originating from the oligonucleotides used, and all of Gpalp. The *STE2-GPA1* fusion construct in pRMHBT4 is
5 transcriptionally regulated by the *GAL1* promoter.

EXAMPLE 2: CONSTRUCTION OF A FUSION BETWEEN THE HUMAN THROMBIN RECEPTOR AND THE YEAST G-ALPHA PROTEIN Gpalp

a) PCR-amplifying thrombin receptor cDNA: a portion of the thrombin gene was PCR amplified from a human lung
10 fibroblast lambda GT10 cDNA library using the following two oligonucleotides:

oligo g) CGGGATCCATAAGCGGCCGCACCCGGGCCCCGCAGGCC

oligo h) GAACGCGTAGTTAACAGCTTTTTGTATATGC

Oligo "g" contains nucleotides 290 to 312 of the
15 thrombin receptor (ThrR) cDNA sequence (GenBank accession # M62424) and sixteen additional bases coding for a BamHI and a NotI restriction site. Oligo "h" contains bases complementary to nucleotides 1477 to 1499 of the ThrR sequence and eight additional
20 nucleotides which include a MluI site. Regions of homology to the ThrR cDNA are in bold type. The PCR product contains bp 291 to 1499 of the human ThrR cDNA sequence, coding for amino acids 22 (arginine) to the COOH-terminal threonine.

25 b) Ligating the human thrombin receptor PCR product into pRMHBT3 as an in-frame fusion to GPA1: the human thrombin PCR product was digested with NotI and MluI and ligated to the 9219 bp NotI/MluI fragment of pRMHBT3 yielding pRMHBT15. The MluI site creates an
30 in-frame fusion of the COOH-terminus of the thrombin receptor (amino acid sequence ...leu-leu-thr) with the NH₂-terminus of Gpalp (amino acids met-gly...), bridged by the tripeptide thr-ag-val as in Example 1d.

c) Creating an in-frame fusion between the Ste2p signal peptide and the NH₂-terminus of the thrombin/Gpa1 fusion: Two oligonucleotides, when annealed, give rise to the double-stranded molecule shown below with overhangs complementary to BamHI and NotI sites.

GATCCATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTAT

GTACAGACTACGCCGTGGAAGTAACTCGTTAGATAAAAATACCGG

This molecule, upon insertion into the BamHI-NotI sites of pRMH15 creates an in frame fusion that encodes the first thirteen amino acids of the Ste2p signal sequence, a bridge glycine (part of the NotI overhang, and the sequence arg-thr-arg-arg... of the thrombin receptor. The above cloning step yielded pRMHBT16.

EXAMPLE 3: TRANSFER OF FUSION CONSTRUCTS OF EXAMPLES 1 AND 2 TO HIGH-COPY VECTORS CONTAINING THE CONSTITUTIVELY ACTIVE PGK PROMOTOR

The fusion constructs in Examples 1 and 2 were placed under the transcriptional control of the PGK1 promoter carried on a yeast 2-micron-plasmid-based vector. A BamHI/NcoI fragment of pRMHBT4 containing the fusion construct and part of the URA3 marker was ligated into the BamHI/NcoI digested pPGK (Kang et al, 1990, Mol. Cell. Biol., 10:2582). Similarly, the BamHI/NcoI fragment of pRMHBT16 containing the ThrR/Gpa1 fusion and part of the URA3 marker was ligated into the BamHI/NcoI digested pPGK. The resulting plasmids were designated pRMHBT18NG and pRMHBT20NG, respectively.

EXAMPLE 4: DISRUPTION OF THE CHROMOSOMAL FAR1 GENE

The FAR1 gene was amplified from yeast genomic DNA using the following primers:

oligo i) CAACATGCAGCCATTTACCG

oligo j) CGCGAGCTCGCCAATAGGTTCTTTCTTAGG

Oligo "i" contains the sequence from residues 34 to 54

of *FAR1* (GenBank accession # M60071) Oligo "j" contains the sequence complementary to nucleotides 2959 to 2980 of the *FAR1* gene and eight additional nucleotides which create a *SacI* restriction site. The amplified sequence extended from nucleotides 34 to 2980. The *FAR1* PCR product was digested with *KpnI* and *SacI*, and ligated into those same sites in the yeast integrating vector pRS306 (Sikorski and Hieter, 1989, *Genetics* 122:19-27). The resulting plasmid was designated p*FAR1*. The *far1* mutation was constructed by deleting an internal 700 bp *XbaI* fragment from p*FAR1*, which removed bp 1917 to 2616, and results in a protein that is missing 153 of its 781 amino acids. The resulting plasmid was designated p*FARX*. The p*FARX* plasmid was used to introduce the *far1* mutation into the chromosome of the haploid yeast strain MS2288 (mat a, *ura3-52*, *leu2-3,112*, *his3D200*, *trp1D1*; M. Rose, Princeton University). p*FARX* was linearized at its single *EcoRI* site (position 2771 of *FAR1*) and used to transform competent MS2288 cells to uracil prototrophy, thereby integrating the p*FARX* plasmid at the *FAR1* locus. Strains in which the plasmid had recombined back out of the chromosome were identified using 5-FOA selection, and *ura* derivatives were screened for retention of the *far1* mutation by PCR analysis using oligos "i" and "j". The *far1* mutants HBS31 and HBS32 (*far1-X200*) exhibited continued cell division in the presence of alpha factor indicating that the mutation functionally disrupted the chromosomal *FAR1* gene.

EXAMPLE 5: DISRUPTION OF THE CHROMOSOMAL *GPA1* GENE

a) Construction of a *FAR1/far1* diploid strain: Strain HBS10 (mat a, *ura3-52*, *leu2-3,112*, *his3A200*, *trp1A1*, *lys2AS738*, *far1-X200*) was mated to MS16 (mat a, *trp1A1*, *ade2-101*) and the resulting strain was sporulated. Segregants from this cross included

TMHY2-14A (mat a, ura3-52, his3 Δ 200, trp1 Δ 1, lys2 Δ S738, ade2-101). TMHY2-14A was then mated to HBS10 and diploids were selected. The resulting diploid strain was designated TMHY2-223D (a/a, ura3/ura3, leu2/LEU2, his3/his3, trp1/trp1, lys2/lys2, far1/FAR1, ADE2/ade2).

b) Engineering the GPA1 disruption construct: the TRP1 gene was amplified from the vector pRS304 (Sikorski and Hieter, 1989, Genetics 122:19-27) by PCR using the following oligos, both with SphI sites (underlined), to yield a 1134 bp fragment containing a functional TRP1 gene:

oligo k) GAATGCATGCGGCATCAGAGCAG

oligo l) GAATGCATGCGGTATTTTCTCCTTACGC

This PCR product was digested with SphI and ligated into the 8386 bp SphI fragment of pRMHBT3. The two SphI sites, separated by 851 bp, are present within the coding sequence of the GPA1 gene in this plasmid. Replacement of this fragment with the TRP1 gene yielded the plasmid pRMHBT10 in which the TRP1 gene is flanked by GPA1 sequences.

c) Disrupting the chromosomal GPA1 locus: pRMHBT10 was digested with MluI and PflMI to liberate a 1887 bp fragment containing the TRP1 gene flanked by GPA1 sequences as described in 5b. This fragment used to transform the diploid strain TMHY2-223D to tryptophan prototrophy. The deletion was confirmed by PCR analysis of several transformants using the GPA1-specific oligos "c" and "d" of Example 1. These strains were given the designation TMHY3D (genotype a/a, ura3/ura3, his3/his3, lys2/lys2, trp1/trp1, ADE2/ade2, FAR1/far1, LEU2/leu2, GPA1/gpa1::TRP1).

d) Genetic confirmation of GPA1 disruption: Five different TRP⁺ transformants (TMHY3D-1, TMHY3D-2,

TMHY3D-3, TMHY3D-5, and TMHY3D-6) were sporulated and tetrads dissected. Representative data for one of the transformants is given below. Four of seven tetrads produced two normal colonies, one small colony, and one non-viable spore (2:1:1). Two tetrads produced two normal colonies and two non-viable spores (2:0:2). One tetrad produced two normal colonies and two small ones (2:2:0). Similar data was obtained for the other four sporulations.

Each tetrad, on non-selective plates, is expected to give only two normally growing colonies (both GPA1⁺). The two others (gpa1⁻) should be slow-growing (gpa1⁻, far1⁻) or nonviable (gpa1⁻, FAR1⁺). All normally-growing colonies should be trp⁻, whereas all the small and inviable colonies should be TRP⁺. Further analysis confirmed that all normally-growing colonies were trp⁻. Ten of these were analyzed by PCR using the GPA1-specific oligos c and d of Example 1, and confirmed that all carry the wild type GPA1 allele. Nine representative slow-growing colonies from each sporulation were analyzed further: All were TRP⁺ indicating that they carried the gpa1 mutation. Six of these were subjected to PCR analysis as above (using oligos "i" and "j" for FAR1), which confirmed that all six are gpa1⁻, far1⁻.

EXAMPLE 6: COMPLEMENTATION OF THE gpa1 MUTATION BY CLONED GPA1

a) Complementation of gpa1 with a full length GPA1 gene: a 1924 bp EcoRI fragment including the entire GPA1 gene (Dietzel and Kurjan, 1987, Cell 50:1001-1010) was amplified from yeast genomic DNA using the following oligos:

oligo m) GGAATTCCACCAATTTCTTTACG

oligo n) GGAATTCGAGATAATACCCTGTCC

The resulting PCR product was ligated into the EcoRI site of the vector pRS316 (Sikorski and Heiter) and

the 2-micron vector YEp352 (Hill et. al., 1986, Yeast 2:163-167). The resulting plasmids were designated p316GPA1 and p352GPA1, respectively. Strain TMHY3D-1 was transformed with both plasmids to uracil prototrophy, and the strains were sporulated. Complementation of the *gpa1* mutation by a plasmid carrying GPA1 should result in 4:0 segregation for viable vs. small or non-viable colonies (assuming the plasmid segregates to all four spores). However, the theoretical 4:0 segregation expected for full complementation would not be always realized since plasmids are lost at some frequency in both the mitotic divisions in the sporulation medium, and in meiosis. The following results were observed: Of 19 tetrads, 9 from p316GPA1 and 10 from p352GPA1 transformants, 11 segregated 4:0 for normal vs. small or nonviable colonies. In all 11, two colonies per tetrad were *trp*⁻ and two were TRP⁺, and all TRP⁺ colonies were URA⁺, indicating that they carried both the *gpa1* mutation and the GPA1-containing plasmid. Four tetrads segregated 3:1 for normal vs. small or nonviable colonies. In all four tetrads, two colonies were *trp*⁻ and one was TRP⁺, and all the TRP⁺ colonies were also URA⁺. These results indicate that the non-viable spore failed to receive a complementing plasmid. Three tetrads segregated 2:2 for normal vs. small or nonviable colonies. In two of these tetrads, the two viable colonies were *trp*⁻ (GPA1⁺), *ura*⁻, suggesting all four spores lacked the complementing plasmid (plasmid was likely lost in the mitotic divisions preceding meiosis). The other segregated 1:1 for *trp*, probably resulting from incomplete dissection. One tetrad segregated two normally-growing colonies, one slow-growing colony and one non-viable colony. All were *ura*⁻ indicating plasmid loss in mitosis, and the medium-sized colony is likely a *far1*, *gpa1* double-mutant which grew better than others

of the same genotype for unknown reasons. Alternatively, this colony may contain a mutation which partially suppresses the *gpa1* mutant phenotype. These results clearly demonstrate that the cloned *GPA1* gene fully complements the chromosomal *gpa1* mutation.

b) Complementation of the *gpa1* mutation by *GPA1* under PGK1 promoter transcriptional control: the plasmid *PRMHBT20NG* (Example 3) was digested with *Bam*HI and *Mlu*I, blunted with Klenow, and religated to yield *PRMHBT43*. This was transformed into *HBS14* (genotype *a/a*, *ura3/ura3*, *his3/his3*, *lys2/lys2*, *trp1/trp1*, *ADE2/ade2*, *FAR1/far1*, *LEU2/leu2*, *GPA1/gpa1::TRP1*) by selection for uracil prototrophy. 21 tetrads from three *URA*⁺ transformants were dissected. Eleven of these segregated 4:0 and nine segregated 2:2 for normal-growing to slow-growing or inviable colonies. All colonies from the eleven 4:0 tetrads were *URA*⁺, whereas all of the growing colonies from the nine tetrads that segregated 2:2 were *trp*⁻, *ura*⁻. That all 4:0 segregants were *URA*⁺ indicates that the plasmid *PRMHBT43* can efficiently complement the chromosomal *gpa1* mutation.

EXAMPLE 7: COMPLEMENTATION OF THE *gpa1* MUTATION BY EXPRESSION OF A *STE2*-*GPA1* CHIMERIC PROTEIN

The plasmid *PRMHBT18NG* encoding a *Ste2p-Gpalp* fusion protein was used to transform *HBS14* to uracil prototrophy. The resulting strain was sporulated and 20 tetrads were dissected. Six segregated 4:0, four 3:1 and ten 2:2 for normal-growing to slow-growing or inviable colonies. All 4:0 segregants were *URA*⁺, which clearly demonstrates that the *Gpa1-Ste2* chimera can rescue the *gpa1* phenotype. Of the 3:1 segregants, three contained one *TRP*⁺, *URA*⁺ colony, strongly suggesting that the non-viable spore was *gpa1* and did not receive the plasmid. Of the ten 2:2 segregants,

all growing colonies were *trp*⁻, and none were *URA*⁺. This provides further evidence that *prMHBT18NG* complements the *gpa1* mutation. One of the four 3:1 segregating tetrads contained two *TRP*⁺ and one *trp*⁻ colonies, suggesting incomplete dissection.

EXAMPLE 8: COMPLEMENTATION OF THE *gpa1* MUTATION BY EXPRESSION OF A THROMBIN RECEPTOR-GPA1 FUSION PROTEIN

The plasmid *prMHBT20NG* was used to transform strain HBS14 to uracil prototrophy. The resulting strain was sporulated, and 19 tetrads were dissected. Five tetrads segregated 4:0, four segregated 3:1 and ten segregated 2:2 for normal growing to slow-growing or inviable spores. All 4:0 segregants were *URA*⁺, which clearly demonstrates that the ThrR-Ste2 chimera can rescue the *gpa1* phenotype. Of the 3:1 segregants, two contained one *TRP*⁺, *URA*⁺ colony, strongly suggesting that the non-viable spore was *gpa1* and did not receive the plasmid. In nine of the ten 2:2 segregants, all growing colonies were *trp*⁻, and none were *URA*⁺. This provides further evidence that *prMHBT20NG* complements the *gpa1* mutation. One of the three 3:1 segregating tetrads contained two *TRP*⁺ and one *trp*⁻ colonies, suggesting incomplete dissection. One of the 2:2 segregating tetrads contained one *TRP*⁺ colony, but it was also *URA*⁺. That a *trp*⁻ colony is "missing" likely reflects incomplete dissection. Thus all growing *TRP*⁺ (*gpa1*⁻) colonies are *URA*⁺ and therefore contain *prMHBT20NG*. These results demonstrate that the ThrR-Ste2p chimera complements the *gpa1* mutant phenotype.

EXAMPLE 9: REPORTER ASSAYS FOR ACTIVATION OF THE MATING PATHWAY

a) Construction of a *lacZ* gene transcriptionally regulated by the mating pathway-specific *FUS1* promoter: The *FUS1* promoter was amplified from yeast genomic DNA by PCR using oligos "o" and "p" shown

below:

oligo o) GCATGCTGCAGGATCGCCCTTTTGGACG

oligo p) GACGTCGACAGAACTTGATGGCTTATATCCTGC

Oligo "o" contains the sequence of nucleotides 1 to 23
5 of *FUS1* (GenBank accession # M17199) and five
additional nucleotides creating a *SphI* restriction
site. Oligo "p" contains the sequence complementary
to residues 232 to 258 of the *FUS1* gene and eight
10 additional nucleotides which create a *SalI* restriction
site. The amplified sequence encompasses nucleotides
1 to 258, and includes a *PstI* site at residue 1 in
FUS1. The *FUS1* promoter was digested with *SalI* and
PstI, and ligated into those same sites in the vector
pUC19 (Yanisch-Perron et. al., 1985, *Gene* 33:103-119).
15 The resulting plasmid was designated pUFS.

The *LacZ* coding sequence was cut from pON831
(obtained from J. Vieira, University of Washington)
using *SalI* and *KpnI*, and this 3.2 kb fragment was
ligated into pUFS digested with the same enzymes. The
20 resulting plasmid, pFus-Lac, contained the *lacZ* coding
sequence under transcriptional control of the *FUS1*
promoter. The *Fus1-lacZ* gene was then moved into
three different yeast vectors: 1) pFus-Lac was
digested with *SphI* and the resulting FUS-lacZ segment
25 was cloned into the *SphI* site within the coding
sequence of the *FAR1* gene in the plasmid pFAR1. The
resulting plasmid (which is an integrating vector
containing *URA3* as its selectable marker) was
designated pFFLz. 2) pFus-Lac was digested with
30 *HindIII* and *KpnI*, and the resulting FUS-lacZ segment
was cloned into the 2-micron vector YEp352. The
resulting plasmid which uses *URA3* as a selectable
marker was designated pYFL3. 3) pFus-Lac was digested
with *PstI* and *KpnI*, and the resulting FUS-lacZ segment
35 was cloned into the integrating vector YIp351 (Hill
et. al., 1986, *Yeast* 2:163-167). The resulting
plasmid which uses *LEU2* as a selectable marker was

designated pLZ351.

The above plasmids were transformed into yeast strains, and the cells were analyzed for their ability to induce beta-galactosidase in response to alpha factor addition to the growth medium. Strain HBS10 transformed with pYFL3 exhibited an alpha factor-independent beta-galactosidase specific activity of 212 nmol/mgmin, and alpha factor-induced activity of 2023 nmol/mgmin, representing a 9.5 fold induction. pPFLz was digested with EcoRI which linearized the plasmid within the FAR1 coding sequence, and this DNA was used to transform strain HBS10 to uracil prototrophy. This integrated the plasmid at the chromosomal FAR1 locus. HBS10::pPFLz exhibited an alpha factor-independent beta-galactosidase activity of 23 nmol/mgmin and alpha factor-induced activity of 735 nmol/mgmin, representing a 32.0 fold induction.

b) Construction of a LYS2 gene transcriptionally regulated by the mating pathway-specific FUS1 promoter: The FUS1 promoter was cut from pUFS using SphI and SalI, and the 266 bp fragment was ligated into the SphI-SalI sites of Ycp50 to make pRMHBT25. LYS2 was PCR-amplified (only coding sequence and 3' untranslated region) from yeast genomic DNA using the following oligonucleotides:

oligo q) CGGCGGTCGACTAATGACTAACGAAAAGG

oligo r) CCCGGGCGCAAGTATTCATTTTAGACCCATGGTGG

Oligo "q" contains the sequence of nucleotides 299 to 312 of LYS2 (GenBank accession # M36287, M14967) and nine additional nucleotides creating a SalI restriction site. Oligo "r" contains the sequence complementary to nucleotides 4822 to 4850 of the LYS2 gene and six additional nucleotides which create a SmaI restriction site.

The 4566 bp LYS2 PCR product was digested with SalI and SmaI, and ligated into the SalI-NruI sites of

pRMHBT25 to generate pRMHBT26, which contains the *LYS2* coding sequence under transcriptional control of the *FUS1* promoter.

The following experiment was performed to verify mating pathway-dependent activation of the *LYS2* gene: HBS10 cells were transformed to uracil prototrophy by pRMHBT26. Transformants were grown to mid-log in ura media, and cells were back-diluted into ura⁻lys⁺ media with or without 5.8mM alpha factor. Growth was measured by OD₆₀₀, but the initial measurement was taken using a Coulter Counter, yielding a starting cell count of 5.18×10^5 /ml. The time point readings (OD₆₀₀) of the cultures were as follows:

	<u>12.0 hrs</u>	<u>18.0 hrs</u>	<u>24.0 hrs</u>
control	0.035	0.050	0.038
alpha factor	0.068	0.244	1.022

HBS10 (without pRMHBT26) did not grow in lys⁻ media. These results clearly demonstrate that strain HBS10/pRMHBT26 exhibits alpha factor-dependent lysine prototrophic growth, which confirms that expression of *Lys2p* is dependent upon mating pathway activation by alpha factor.

EXAMPLE 10: ALPHA FACTOR-DEPENDENT ACTIVATION OF THE MATING PATHWAY BY THE STE2-GPA1 FUSION IN *gpa1* CELLS

Examples 7 and 8 show that the Gpalp domain of the Ste2-Gpa1 fusion construct functionally complements the chromosomal *gpa1* mutation. To determine if Gb and G dissociate from the Gpa1 domain (Ga) of the Ste2-Gpa1 fusion protein in an alpha factor-dependent manner and therefore propagate the mating pathway activation signal, the following experiments were performed:

a) Shmoo formation assay: Strain 9A/pRMHBT18NG (a haploid segregant of HBS14 carrying the plasmid

5 pRMHBT18NG, whose genotype is *mat a*, *ade2-101*, *ura3-52*, *leu2,3-112*, *his3D200*, *trp1D1*, *lys2-DS738*, *far1-X200*, *gpa1::TRP1* (the *gpa1* mutation is complemented by the *URA3*-containing plasmid pRMHBT18NG)) was grown to mid-log in *ura* media, and alpha factor was added to 5.8mM. Microscopic examination after 5.0 hours clearly showed that more than 70.0% of the pheromone-treated cultures were shmooed, while less than 10.0% of the no-alpha factor controls were shmooed. These results demonstrates alpha factor-dependent dissociation of the Gb and Gg subunits from the Gpalp domain of the Ste2-Gpa1 fusion protein, resulting in subsequent activation of the mating response pathway.

15 b) LYS2 prototrophy assay: To change the selectable marker from *URA3* to *HIS3*, the 6159 bp *ApaI*-*ClaI* fragment of pRMHBT26 containing the *FUS1* promoter-*LYS2* gene fusion was ligated into the *ApaI*-*ClaI* sites in pRS313 (Sikorski and Hieter) to generate pRMHBT41. Strain 9A/pRMHBT18NG was transformed to histidine prototrophy with pRMHBT41, resulting in strain 9A/pRMHBT18NG/pRMHBT41. Cells were grown to mid-log in *ura**his* media, at pH 6.5 and 4.0. 9A/pRMHBT18NG controls were grown similarly in *ura* media. The cells were washed three times with sterile water before being diluted into the experimental (*lys*-) media. Each group of cells was back-diluted into two aliquots - one of which contained 5.8mM alpha factor.

In *ura**his**lys* media:

1. 9A/pRMHBT18NG/pRMHBT41, pH 4.0
2. 9A/pRMHBT18NG/pRMHBT41, pH 4.0 + alpha factor
3. 9A/pRMHBT18NG/pRMHBT41, pH 6.5
4. 9A/pRMHBT18NG/pRMHBT41, pH 6.5 + alpha factor

In *ura**lys* media:

5. 9A/pRMHBT18NG pH 4.0
6. 9A/pRMHBT18NG pH 4.0 + alpha factor

7. 9A/pRMHBT18NG pH 6.5

8. 9A/pRMHBT18NG pH 6.5 + alpha factor

Cell growth was monitored by OD₆₀₀. Each aliquot received the same number of cells (volumetrically).

5 The time point readings were as follows:

	<u>expt</u>	<u>6 hrs</u>	<u>12 hrs</u>	<u>24 hrs</u>	<u>30 hrs</u>	<u>36 hrs</u>
	1.	0.000	0.000	0.045	0.171	0.582
	2.	0.000	0.035	0.570	2.010	3.284
	3.	0.000	0.007	0.023	0.039	0.058
10	4.	0.002	0.021	0.132	0.500	0.682
	5.	0.003	0.014	0.016	0.021	0.019
	6.	0.003	0.010	0.016	0.018	0.018
	7.	0.006	0.017	0.016	0.021	0.022
	8.	0.002	0.006	0.014	0.020	0.009

15 The results shown above, like those in 9b, clearly demonstrate that strain 9A/pRMHBT18NG/pRMHBT41 exhibits alpha factor-dependent lysine prototrophic growth at pH 4.0 and pH 6.5, which confirms that expression of Lys2p is dependent upon mating pathway

20 activation (by alpha factor). The very slow growth seen in "1" is most likely due to basal activity of the *FUS1* promotor. That we did not see slow growth in "3" probably reflects the fact that the yeast pH optima for growth is less than 4, and at 6.5 they are

25 sufficiently stressed as to be unable to support lysine prototrophy from the basal activity of the *FUS1* promotor. Alpha factor-dependent lysine-prototrophic growth demonstrates that the Ste2p-Gpalp fusion protein activates the mating pathway in a *gpa1*

30 background. Importantly, the mating pathway is not constitutively activated in the *gpa1* strain 9A/pRMHBT18NG/pRMHBT41 since lysine prototrophy is

alpha factor-dependent. This further supports the conclusion that the Gpalp domain of the Ste2-Gpalp fusion protein correctly associates with the Gb and Gg subunits. Also, the mating pathway can be effectively
5 activated at pH 6.5, which more closely resembles physiological conditions for mammalian receptors. Additionally, the higher pH preferably pH 6 to 7.5 reduces background prototrophy due to basal activity of the FUS1 promotor ("1" vs. "3"). Thus, by
10 selecting for lysine prototrophy, we can identify cells whose mating pathways are initiated via ligand-dependent activation of mammalian receptors fused to Gpalp.

c) Lac Z reporter assay: pLZ351 (see 9 above) was
15 digested with BstEII to linearize the plasmid within the LEU2 sequence, and then this DNA was used to transform strain 9A/pRMHBT18NG to leucine prototrophy. Strain 9A/pRMHBT18NG is a haploid segregant of HBS14 carrying the plasmid pRMHBT18NG, whose genotype is mat
20 a, ade2-101, ura3-52, leu2,3-112, his3D200, trp1D1, lys2-DS738, far1-X200, gpal::TRP1 (the gpal mutation is complemented by the URA3-containing plasmid pRMHBT18NG). The resulting strain is designated 9ALZ/pRMHBT18NG. Cells were grown to mid-log in ura
25 media, and diluted to an OD₆₀₀ of approximately 0.3 in the same media. Cells were treated with alpha factor at 5.8mM and incubated at 30°C for 3.0 hours. Cell lysates from alpha factor treated and control cells were prepared and assayed for beta-galactosidase
30 specific activity (Rose et. al., Methods in Yeast Genetics: A Laboratory Course Manual, 1990, CSH Laboratory Press). This yielded specific activities of 18.1 nmol/min/mg for untreated cells, and 122.2 nmol/min/mg for alpha factor-treated cells. This is a
35 6.8-fold induction of activity upon alpha factor treatment, and clearly demonstrates alpha factor-

dependent activation of the yeast mating pathway through the Ste2p-Gpalp fusion protein.

EXAMPLE 11: ALPHA FACTOR-DEPENDENT ACTIVATION OF THE YEAST MATING PATHWAY BY THE STE2-GPA1 FUSION IN *ste2* CELLS

a) Disruption of the STE2 gene in HBS32 cells: The STE2 gene was PCR amplified from genomic DNA using the following two oligonucleotides:

s) AGTGCGGCCGCAAGCTTATGTCTGATGCGGCTCCTTCATTG

t) ACGCGTTCTAGATCATAAATTATTATTATCTTCAGTCCAGAAC

Oligonucleotide "s" contains the sequence from bp 534 to 557 of STE2 (GenBank accession # M24335) and seventeen additional nucleotides creating NotI and HindIII restriction sites. Oligonucleotide "t" contains the sequence complementary to bp 1800 to 1832 of the STE2 gene and ten additional nucleotides which create MluI and XbaI restriction sites. The resulting 1295 bp PCR product was digested with NotI and XbaI and ligated into pBluescript (Stratagene) cut with the same enzymes. An internal NsiI fragment of the STE2 gene (at positions 1148 and 1436) was deleted by digestion with NsiI and religation, creating a frameshift mutation in addition to the deletion. The resulting plasmid was digested with HindIII and XbaI and the 1005 bp fragment with the STE2 deletion mutation was ligated into the yeast integrating vector pRS306 (Sikorski and Hieter *Genetics* 122:19 (1989)). This mutant gene was used to replace wild type STE2 by the two step method. The deletion plasmid was linearized within STE2 at the HpaI site and integrated into HBS32 by selection for uracil prototrophy. Strains in which the plasmid had recombined back out of the chromosome were identified using 5-FOA selection, and these ura⁻ derivatives were screened for

the *ste2* mutation by PCR analysis using oligonucleotides s and t. One resulting strain with a such a *ste2* deletion was designated HBS12. The FUS1-LACZ reporter construct was integrated into HBS12 as described in Example 10C to make the strain HBS12LZ (leu2::LEU2-FUS1-LACZ).

b) Overexpression of Ste2p rescues the *ste2* phenotype. Strain HBS12LZ was transformed to uracil prototrophy with pRMHBT45, which is a 2-micron *URA3*-marked vector containing the coding sequence of *STE2* under transcriptional control of the PGK promoter, with termination signals from the *GPA1* gene (note that this was not a fusion construct, and no *GPA1* coding sequences were present). HBS12LZ/pRMHBT45 was grown to mid-log phase in ura⁻ media, and alpha-factor was added to 5.8 mM. After four hours of incubation at 30.0°C on a roller drum, microscopic examination revealed that over 80.0% of the treated cells were shmoos, while shmoos were undetectable in an untreated control culture. This result clearly shows that the *STE2* construct pRMHBT45 carries a functional *STE2* gene. Beta-galactosidase assays confirmed this conclusion, as follows.

<u>Strain</u>	<u>Miller Units</u>
HBS12LZ/pRMHBT45	2.44 +/- .052
HBS12LZ/pRMHBT45 + α -factor	90.81 +/- 1.29

$p = <10^{-6}$ (ANOVA- Duncan's post-hoc test)

- 5 These results clearly show mating factor-dependent activation of the FUS1p-LACZ reporter, and confirms that pRMHBT45 carries a functional *STE2* gene.

- 10 c) The Ste2p domain of the Ste2p-Gpal chimera is functional: To determine if the Ste2p domain of the Ste2p-Gpalp fusion protein is functional (able to bind alpha factor and transmit the binding signal to Gpal), the following experiment was performed. Strain HBS12LZ was transformed to uracil prototrophy with pRMHBT18NG, which carries the fusion construct, and
- 15 the resulting strain, HBS12LZ/pRMHBT18NG was examined for alpha factor-dependent shmoo formation. Cells were grown to mid-log phase in ura⁻ media, and alpha factor was added to 5.8 mM. After four hours (post-addition) of incubation at 30.0°C on a roller drum,
- 20 >50.0% of the alpha factor treated cells had formed shmoos, while no shmoos were detected in untreated controls. These results clearly show that the Ste2p domain of the Ste2p-Gpalp fusion protein is functional. Additionally, a quantitative beta-

galactosidase assay was performed on these cultures as described previously:

	<u>Strain</u>	<u>Miller Units</u>
	HBS12LZ/pRMHBT18NG	5.5 +/- .098
5	HBS12LZ/pRMHBT18NG + α -factor	70.97 +/- 1.90

$p = <10^{-6}$ (ANOVA, Duncan's post-hoc test)

These results clearly show mating factor-dependent activation of the FUS1p-LACZ reporter, and confirms that the Ste2p domain of the Ste2p-Gpalp chimera from pRMHBT18NG is functional and rescues the *ste2*-deletion phenotype.

EXAMPLE 12: ENHANCED ACTIVATION OF THE MATING PATHWAY BY THE STE2-GPA1 FUSION IN *ste2*, *gpa1* CELLS

a) Deletion of chromosomal STE2 in strain 9ALZ: The strain 9ALZ/pRMHBT18NG with a chromosomal *gpa1* mutation was transformed to lysine prototrophy with pRMHBT44 to remove the URA3 marker of pRMHBT18NG and replace it with a LYS2 marker. pRMHBT44 is functionally equivalent to pRMHBT43 (GPA1 under PGK promoter transcriptional control), except it has a LYS2 marker. The strain 9ALZ/pRMHBT44 was identified by 5-FOA counter-selection against the URA3-containing plasmid pRMHBT18NG. This strain was used for disruption of the STE2 gene by the two step method using URA3, as in example 11. The new *ste2*, *gpa1* strain was designated 9ALZAGS/pRMHBT44. A "plasmid shuffle" was then performed to replace pRMHBT44 with pRMHBT18NG carrying the STE2-GPA1 fusion construct. Strain 9ALZAGS/pRMHBT44 was transformed to uracil prototrophy with pRMHBT18NG. URA⁺ cells were then

grown to saturation in ura⁻ media, and cells that had lost pRMHBT44 were selected for by growth on ura⁻ plates with 5.0% α -aminoadipic acid (α -aminoadipic acid is lethal to LYS⁺ cells, and selects against pRMHBT44). A similar plasmid shuffle was also performed to replace pRMHBT44 with pRMHBT20NG. The resulting strains were designated 9ALZAGS/pRMHBT18NG and 9ALZAGS/pRMHBT20NG, respectively.

b) Activation of the mating pathway by the Ste2p-Gpalp fusion protein in *gpa1, ste2* cells: This was done by demonstrating that the Ste2p-Gpalp fusion can transduce the α -factor binding signal to cause activation of the mating pathway. Strain 9ALZAGS/pRMHBT18NG was grown to mid-log phase in ura⁻ media. Cells were back-diluted to 0.2 OD₆₀₀, and α -factor was added to 5.8 mM. Cells were grown at 30.0°C on a roller drum for an additional 4.0 hours, then examined by light microscopy and prepared for beta-galactosidase assays as described previously. Two independent experiments were performed. In both, over 90.0% of the treated cells had formed shmoo after 4.0 hours, while less than 5.0% were shmoo-like in the untreated (control) cultures. Quantitative beta-galactosidase assays were performed as described previously, providing the following results in two separate experiments:

61

	<u>Strain</u>		<u>Exp. #</u>
	<u>Miller Units</u>		
	9ALZAGS/pRMHBT18NG	1	8.59 +/- .195
		2	12.76 +/- .104
5	9ALZAGS/pRMHBT18NG + α -factor	1	41.40 +/- .67
		2	80.83 +/- .433

p= $<10^{-6}$ for both experiments (ANOVA, Duncan's post-hoc test)

10 These results clearly show that the Ste2p-Gpa1 chimera
can complement the deletion of both *ste2* and *gpa1*, and
can transduce the alpha factor binding signal to
initiate mating pathway activation. Reporter gene
activity is enhanced 4.8 fold and 6.3 fold in the two
15 experiments indicating that the mating pathway is
strongly activated by alpha factor binding. Note that
the basal levels without alpha factor are higher in
the experiments described in this and the following
sections (and in similar experiments in Examples 10c,
20 13 and 14) than in cells without the *gpa1* mutation
(Example 9a). This is probably because the *gpa1*
mutation is not completely complemented by any of the
constructs, leading to a low basal level of activation
of the mating pathway and consequent low levels of
25 beta-galactosidase activity.

c) Activation of the mating pathway by Ste2p and Gpalp
expressed separately in *ste2 gpa1* cells: As a control
for the previous experiment, Ste2p and Gpalp were
expressed separately from the same promoter and vector

in the same yeast strain used to express the fusion protein. 9ALZAGS/pRMHBT44/pRMHBT45 cells were grown to mid-log phase in ura⁻ media. α -factor was added to 5.8 mM, and the cells were assayed for beta-galactosidase activity after incubation for four hours at 30°C on a roller drum. Cells were also observed via light microscopy after 4.0 hours of incubation, and no shmooos were detectable in either the treated or non-treated control cultures. The beta-galactosidase assay data is shown below:

<u>Strain</u>	<u>Miller Units</u>
9ALZAGS/44/45	8.00 +/- 0.08
9ALZAGS/44/45 + α -factor	8.2 +/- 0.10

While there is a significant difference between the two cultures ($p = .026$, ANOVA, Duncan's post-hoc test), it is very small. Thus, we conclude that the mating pathway is only weakly activated (2.4% stimulation due to mating pheromone) in response to alpha factor in cells expressing Gpalp and Ste2p from the same promoter and vector as the fusion protein in the previous section "b". In contrast, the fusion protein expressed in the same strain from the same promoter and vector causes, in two experiments, a 4.8-fold and a 6.3-fold enhancement of reporter gene activity. Assuming that levels of Ste2p and Gpalp proteins in this experiment are comparable to levels of the Ste2p-Gpalp fusion protein in the experiments described in section "b", the efficiency of coupling between Ste2p and Gpalp when fused is greater by two orders of magnitude than when separate. This experiment is a more appropriate control for the fusion protein than comparing the efficiency to the separated components in a wild type STE2, GPA1 cell, since expression of the two genes in the wild type cell has been optimized by evolution for maximal sensitivity to mating factor.

EXAMPLE 14: THROMBIN-DEPENDENT ACTIVATION OF THE YEAST MATING PATHWAY BY THE HUMAN THROMBIN RECEPTOR-GPA1 FUSION PROTEIN IN *ste2 gpa1* CELLS

9ALZAGS cells were transformed with the thrombin
5 construct pRMHBT20NG by the plasmid shuffle method
described in Example 12a. These cells were grown to
mid-log phase in ura media buffered at pH 7.0. Human
thrombin was added to 71.4 units/ml media, and the
10 cells were incubated for four hours at 30°C on a roller
drum. Crude extracts were then prepared as described
and beta-galactosidase assays were performed. The
results are shown below:

	<u>Strain</u>	<u>Miller Units</u>
	9ALZAGS/20NG	9.49 +/- 0.22
15	9ALZAGS/20NG + thrombin	12.97 +/- 0.046

These measurements are significantly different ($p=$
1.215 x 10⁻⁵; ANOVA, Duncan's post-hoc test), and
indicate that there was a 37% stimulation of beta-
galactosidase activity in response to thrombin. While
20 not as great in magnitude as activation of the pathway
by alpha factor binding to the Ste2p-Gpalp chimera
(Example 12b), these results are consistent with the
results in Example 13, and show that the two
components of the Thrombin Receptor-Gpalp fusion
25 protein can measurably couple with each other and to
the yeast mating pathway. Further modification of
the Gpalp domain of the chimera by methods such as
that described in Example 15 should enable greater
efficiency of coupling. In addition, the host strain
30 can also be modified by random mutagenesis to reduce
the background activation and to enhance the induction
of the mating pathway. Mutants that provide
hypersensitivity to mating factor are known (e.g. Chan
et al, 1982, Mol. Cell. Biol. 2:21)

35 EXAMPLE 15: MUTAGENESIS OF THE Gpalp DOMAIN OF A

FUSION PROTEIN TO ENABLE COUPLING OF THROMBIN RECEPTOR
ACTIVATION TO THE MATING PATHWAY

a) constructing a library of mutations:
oligonucleotides "c" and "d" described in example 1
5 are used to amplify the GPA1 gene from a wild type
plasmid copy under PCR conditions shown to introduce
mutations at a frequency of 6.6 per 1000 bases
(Cadwell and Joyce, 1994, in "PCR and its
applications", CSHL Press, pp S136). This method
10 introduces transition and transversion mutations but
not insertion or deletion mutations, thus maintaining
the reading frame but randomizing the amino acid
sequence. The method also has no significant sequence
bias. The amplification product that contains
15 individual molecules with single or multiple mutations
is digested at the MluI and PflMI sites present in the
two primers. The plasmid pRMHBT20, which encodes the
thrombin receptor fused in frame with Gpalp, is
digested with MluI and PflMI to release GPA1, and the
20 fragment with the thrombin receptor is purified and
ligated to the digested PCR product, and transformed
into competent E. coli with the highest transformation
efficiency that is commercially available. Each
transformant carries a different mutation of the Gpalp
25 domain of the fusion protein. The entire
transformation mix is plated on large plates, and
plasmids are isolated from these plate cultures. The
maximum number of recombinants are needed to obtain
the largest collection of mutations, and the above
30 steps are repeated and the plasmid preparations pooled
in proportion to the number of mutations represented
in each pool until at least 10^7 mutants are included in
the library. The construction and use of mutant
libraries of GPA1 have been described previously
35 (Stone and Reed (1990) Mol. Cell. Biol. 10:4439;
Kurjan et al (1991) Genes Dev. 5:475).

b) screening the library for functional Gpalp domains:

The library is screened first for mutants that will still enable complementation of the *gpa1* mutation, which will eliminate all mutants that do not enable interaction with G-beta/gamma. For this, a diploid yeast strain of genotype *gpa1/gpa1* is constructed by mating haploid *gpa1* strains of opposite mating type in which the *gpa1* mutation is complemented by a plasmid carrying *GPA1*. For example, the strain 9ALZ carrying pRMHBT44 (with a *LYS2* selectable marker) is mated to any of the *URA+* segregants in Example 7a or 7b that are of the alpha mating type. Both strains carry the reporter construct *FUS1-LACZ* integrated at the *LEU2* locus (*leu2::LEU2-FUS1p-LACZ*). Diploids are selected on *ura-*, *lys-* plates. The two plasmids are then eliminated by counterselection with 5-FOA and alpha-aminoadipate, which is possible because *GPA1* is required for growth only in haploids and not diploids.

This strain is transformed with the mutant library so that at least 10^6 *URA+* transformants are obtained, if necessary by repeated transformation experiments. The population of transformants is then sporulated, and random spores are germinated to yield at least 10^5 individual colonies by standard genetic or chemical methods for random spore analysis (Rose et al, *Methods in Yeast Genetics: A Laboratory Course Manual*. c. 1990 by Cold Spring Harbor Laboratory Press.). Only spores in which the *Gpalp* domain of the fusion can complement the *gpa1* mutation can grow, thus selecting for mutants with *Gpalp* domains that can interact with G-beta/gamma.

c) screening for functional coupling of thrombin receptor activation to the mating pathway: this is achieved by growing the mutants selected from the previous step in the presence of thrombin and the dye X-gal, which is a substrate of the reporter *lacZ* gene. Functional coupling is selected for by induction of

- beta galactosidase, and consequent blue color formation. Note that because the reporter gene is present at both LEU2 loci in the diploid, all haploid segregants will have a functional reporter construct.
- 5 Growth of such cells on plates containing thrombin and the dye X-gal causes blue color formation in colonies in which functional coupling is present between the receptor and Gpal domains. Others will remain white.

CLAIMS.

What is claimed is:

1. A method for creating a yeast cell which expresses a fusion protein comprising a seven-transmembrane receptor protein of mammalian or fungal origin operatively linked at its carboxy- terminus to the amino terminus of a G_α protein of a non-mammalian organism so as to activate a pheromone-induced signal transduction pathway in said yeast cell upon binding of a ligand for said receptor to the receptor, which comprises:
 - i) creating a DNA fragment encoding the seven-transmembrane receptor and the G_α protein fused at their respective carboxy- and amino-terminal ends or creating a DNA fragment encoding the seven-transmembrane receptor fused at its carboxy-terminal end to the amino terminal end of a linker peptide and a G_α protein fused at its amino terminal end to the carboxy-terminal end of said linker peptide, to obtain a DNA fragment encoding a fusion protein;
 - ii) adding to the DNA fragment encoding said fusion protein additional nucleotides that encode additional amino acids effective for directing the fusion protein to the plasma membrane of said yeast upon expression of said fusion protein to obtain a DNA fragment encoding a plasma membrane-targeted fusion protein;
 - iii) operatively linking said DNA fragment encoding a plasma membrane-targeted fusion protein to a promoter effective in said yeast for expressing said plasma membrane-targeted fusion protein from said DNA fragment encoding a plasma membrane-targeted fusion protein in said yeast to form a fusion protein expression construct; and
 - iv) transforming said yeast with said fusion protein expression construct; and

v) isolating a cell of yeast which expresses said fusion protein as a part of its plasma membrane.

2. The method of claim 1, wherein said yeast is
5 *Saccharomyces cerevisiae*.

3. The method of claim 2, wherein said G_s protein is encoded by the *Saccharomyces cerevisiae* gene GPA1.

4. The method of claim 2, wherein said seven-transmembrane receptor protein is selected from the
10 group consisting of adenosine receptor A1, adenosine receptor A2, adrenergic receptor A2B, adrenergic receptor α -1A, adrenergic receptor α -1B, adrenergic receptor α -2A, adrenergic receptor α -2B, adrenergic receptor α -2C, adrenergic receptor β -1, adrenergic
15 receptor β -1, adrenergic receptor β -3, amyloid protein precursor, angiotensin II receptor type 1, antidiuretic hormone receptor, bradykinin receptor, cannabinoid receptor, chemokine C-C (mip-1/RANTES) receptor, cholecystokinin receptor, complement C5a
20 (anaphylotoxin) receptor, dopamine receptor D1, dopamine receptor D2, dopamine receptor D3, dopamine receptor D4, dopamine receptor D5, endothelin receptor A, endothelin receptor B, f-met-leu-phe receptor, follicle stimulating hormone receptor, glutamate
25 receptor (metabotropic), gonadotropin-releasing factor receptor, growth hormone releasing hormone receptor, histamine H2 receptor, hydroxytryptamine (serotonin) receptor 1A, hydroxytryptamine (serotonin) receptor 1B, hydroxytryptamine (serotonin) receptor 1C,
30 hydroxytryptamine (serotonin) receptor 1D, hydroxytryptamine (serotonin) receptor 1E, hydroxytryptamine (serotonin) receptor 2, insulin-like growth factor II receptor, interleukin 8 receptor A, interleukin 8 receptor B, lutenizing hormone/chorionic

gonadotropin receptor, *mas* proto-oncogene, melanocyte stimulating hormone receptor, muscarinic acetylcholine receptor m1, muscarinic acetylcholine receptor m2 muscarinic acetylcholine receptor m3, muscarinic acetylcholine receptor m4, muscarinic acetylcholine receptor m5, neuropeptide Y receptor, opioid- δ receptor, opioid- κ receptor, oxytocin receptor, platelet activating factor receptor, rhodopsin receptor, somatostatin receptor 1, somatostatin receptor 2, somatostatin receptor 3, substance K (neurokinin A) receptor, substance P (NK1) receptor, thrombin receptor, thromboxane A2 receptor, thyroid stimulating hormone receptor and vasoactive intestinal peptide receptor.

5. The method of claim 2, wherein step iv) is performed using a vector that is an autonomously replicating vector.

6. The method of claim 2, wherein step iv) is performed using a vector that is a chromosomally-integrating vector.

7. A method for creating a yeast cell which expresses a fusion protein comprising a seven-transmembrane receptor protein of mammalian or fungal origin operatively linked at its carboxy-terminus to the amino terminus of a G_o protein of a non-mammalian organism so as to activate a pheromone-induced signal transduction pathway in said yeast cell upon binding of a ligand for said receptor to the receptor, which comprises:

- i) creating a DNA fragment encoding the seven-transmembrane receptor and the $G\alpha$ protein fused at their respective carboxy- and amino-terminal ends to obtain a DNA fragment encoding a fusion protein;
- ii) adding to the DNA fragment encoding said

fusion protein additional nucleotides that encode additional amino acids effective for directing the fusion protein to the plasma membrane of said yeast upon expression of said fusion protein to obtain a
5 DNA fragment encoding a plasma membrane-targeted fusion protein;

iii) operatively linking said DNA fragment encoding a plasma membrane-targeted fusion protein to a promoter effective in said yeast for
10 expressing said plasma membrane-targeted fusion protein from said DNA fragment encoding a plasma membrane-targeted fusion protein in said yeast to form a fusion protein expression construct;

iv) mutating the gene of said yeast homologous
15 in function to the *FAR1* gene of *Saccharomyces cerevisiae* to inactivate the protein homologous in function to Far1p of *Saccharomyces cerevisiae* in said yeast;

v) constructing a diploid cell of said yeast,
20 wherein said diploid cell has one wild-type gene homologous in function to the *GPA1* gene of *Saccharomyces cerevisiae* and one inactivated copy of said gene homologous in function to the *GPA1* gene of *Saccharomyces cerevisiae*;

vi) transforming said diploid cell of said yeast
25 with the fusion protein expression construct; and

vii) isolating a cell of yeast which expresses said fusion protein as a part of its plasma membrane.

30 8. The method of claim 7, wherein said yeast is *Saccharomyces cerevisiae*, said gene of said yeast homologous in function to the *FAR1* gene of *Saccharomyces cerevisiae* is *FAR1* of *Saccharomyces cerevisiae* and said gene homologous in function to the
35 *GPA1* gene of *Saccharomyces cerevisiae* is the *GPA1* gene of *Saccharomyces cerevisiae*.

9. The method of claim 8, wherein said seven-transmembrane receptor protein is selected from the group consisting of adenosine receptor A1, adenosine receptor A2, adrenergic receptor A2B, adrenergic receptor α -1A, adrenergic receptor α -1B, adrenergic receptor α -2A, adrenergic receptor α -2B, adrenergic receptor α -2C, adrenergic receptor β -1, adrenergic receptor β -1, adrenergic receptor β -3, amyloid protein precursor, angiotensin II receptor type 1, antidiuretic hormone receptor, bradykinin receptor, cannabinoid receptor, chemokine C-C (mip-1/RANTES) receptor, cholecystokinin receptor, complement C5a (anaphylotoxin) receptor, dopamine receptor D1, dopamine receptor D2, dopamine receptor D3, dopamine receptor D4, dopamine receptor D5, endothelin receptor A, endothelin receptor B, f-met-leu-phe receptor, follicle stimulating hormone receptor, glutamate receptor (metabotropic), gonadotropin-releasing factor receptor, growth hormone releasing hormone receptor, histamine H2 receptor, hydroxytryptamine (serotonin) receptor 1A, hydroxytryptamine (serotonin) receptor 1B, hydroxytryptamine (serotonin) receptor 1C, hydroxytryptamine (serotonin) receptor 1D, hydroxytryptamine (serotonin) receptor 1E, hydroxytryptamine (serotonin) receptor 2, insulin-like growth factor II receptor, interleukin 8 receptor A, interleukin 8 receptor B, lutenizing hormone/chorionic gonadotropin receptor, *mas* proto-oncogene, melanocyte stimulating hormone receptor, muscarinic acetylcholine receptor m1, muscarinic acetylcholine receptor m2, muscarinic acetylcholine receptor m3, muscarinic acetylcholine receptor m4, muscarinic acetylcholine receptor m5, neuropeptide Y receptor, opioid- δ receptor, opioid- κ receptor, oxytocin receptor, platelet activating factor receptor, rhodopsin receptor, somatostatin receptor 1, somatostatin receptor 2, somatostatin receptor 3, substance K

(neurokinin A) receptor, substance P (NK1) receptor, thrombin receptor, thromboxane A2 receptor, thyroid stimulating hormone receptor and vasoactive intestinal peptide receptor.

5 10. The method of claim 8, which further comprises step:

 viii) sporulating a transformant obtained from step vii) and isolating a cell having a genotype analogous to a *gpa1*, *far1* genotype.

10 11. The method of claim 8, which further comprises steps:

 viii) creating a second DNA construct comprising a promoter for a gene homologous in function to the *FUS1* gene of *Saccharomyces cerevisiae* operatively
15 linked to a DNA fragment encoding a protein for measuring the activation of said promoter;

 ix) sporulating transformants obtained from step vii) to isolate a haploid cell having a genotype analogous to a *gpa1*, *far1* genotype;

20 x) transforming the haploid cell obtained in step ix) with the second DNA construct of step viii); and

 xi) isolating a haploid cell of yeast having a genotype analogous to a *gpa1*, *far1* genotype which
25 expresses said enzyme under the control of the promoter for the gene homologous in function to the *FUS1* gene of *Saccharomyces cerevisiae* and which expresses said fusion protein as a part of its plasma membrane.

30 12. The method of claim 11, wherein said protein for measuring the activity of said promoter is an enzyme for which a colorimetric assay can be used to measure the catalytic activity of the enzyme, for which an immunoassay can be used to measure the amount

of said protein present in a sample or for which a biochemical selection can be performed to assay expression of the protein.

5 13. The method of claim 12, wherein said protein is selected from the group consisting of β -galactosidase, glucuronidase, green fluorescence protein, luciferase, alkaline phosphatase and invertase.

10 14. A cell of a yeast created according to the method of claim 1.

15 15. A cell of a yeast created according to the method of claim 7.

16 16. A cell of a yeast created according to the method of claim 10.

17 17. A cell of a yeast created according to the method of claim 11.

20 18. A haploid cell of a yeast having a genotype analogous to *gpa1*, *far1* of *Saccharomyces cerevisiae*, which expresses as a part of the plasma membrane of said cell a fusion protein comprising a seven-transmembrane receptor protein attached by its carboxy-terminus to the amino terminus of a G_a protein of said yeast.

25 19. A cell according to claim 18, which is a cell of *Saccharomyces cerevisiae* having a genotype *gpa1*, *far1*.

20 20. A haploid cell of a yeast having a genotype analogous to *gpa1*, *far1* of *Saccharomyces cerevisiae*, which expresses as a part of the plasma membrane of

5 said cell a fusion protein comprising a seven-transmembrane receptor protein attached by its carboxy-terminus to the amino terminus of a G_o protein of said yeast and which further expresses a reporter gene for measuring the activity of a promoter of a gene homologous in function the *FUS1* gene of *Saccharomyces cerevisiae* under the control of said promoter.

10 21. A cell according to claim 20, which is a cell of *Saccharomyces cerevisiae* having a genotype *gpa1*, *far1* and wherein said promoter of a gene homologous in function the *FUS1* gene of *Saccharomyces cerevisiae* is a promoter of the *FUS1* gene of *Saccharomyces cerevisiae*.

15 22. A cell according to claim 21, wherein said reporter gene encodes a protein that is an enzyme for which a colorimetric assay can be used to measure the catalytic activity of the enzyme, for which an immunoassay can be used to measure the amount of said
20 protein present in a sample or for which a biochemical selection can be performed to assay for expression of the protein.

25 23. A cell according to claim 22, wherein said protein is selected from the group consisting of β -galactosidase, glucuronidase, green fluorescence protein, luciferase, alkaline phosphatase and invertase.

24. A method for screening a compound for receptor agonist activity which comprises:
30 i) contacting a yeast cell according to claim 20 with the said compound;
ii) measuring the amount of expression of said

75

reporter gene, to determine the activity of the promoter homologous in function to the promoter of the *FUS1* gene of *S. cerevisiae*; and

- 5 iii) comparing the activity of said promoter in said yeast cells contacted with said compound to the activity of said promoter in said yeast cells not contacted with said compound;
wherein a compound is determined to be an agonist of said receptor if the activity of the promoter is
10 higher in the cell contacted with said compound than in the cell not contacted with said compound.

25. A method for screening a compound for receptor antagonist activity which comprises:

- 15 i) contacting a yeast cell according to claim 20 with the said compound and with a ligand for said receptor;

- 20 ii) measuring the amount of expression of said reporter gene, to determine the activity of said promoter homologous in function to the promoter of the *FUS1* in said yeast cell; and

- 25 iii) comparing the activity of said promoter in said yeast cells contacted with said compound and said ligand to the activity of said promoter in said yeast cells contacted with said ligand but not contacted with said compound;

- wherein a compound is determined to be an antagonist of said receptor if the activity of the promoter is lower in the cell contacted with said compound and said ligand than in the cell contacted with said
30 ligand and not contacted with said compound.

26. A recombinant DNA molecule encoding a fusion protein comprising a first polypeptide means for binding to a ligand and a second polypeptide means for binding to a yeast $G\beta\gamma$ complex, wherein said first
35 polypeptide means is attached by its carboxyl terminus

to the amino terminus of said second polypeptide means, and wherein said fusion protein productively interacts with the pheromone-induced signal transduction pathway of said yeast.

5 27. The recombinant DNA of claim 26 wherein said second polypeptide means is the Gpal protein of *Saccharomyces cerevisiae* or a mutant thereof which is selected by activation of the *S. cerevisiae* pheromone-induced signal transduction pathway upon ligand
10 binding to said first polypeptide means.

28. The recombinant DNA of claim 26, wherein said first polypeptide means is a receptor having seven transmembrane domains.

15 29. The recombinant DNA of claim 27, wherein said first polypeptide means is a receptor having seven transmembrane domains.

20 30. The recombinant DNA of claim 28, wherein said first polypeptide means is a protein of a human selected from the group consisting of adenosine receptor A1, adenosine receptor A2, adrenergic receptor A2B, adrenergic receptor α -1A, adrenergic receptor α -1B, adrenergic receptor α -2A, adrenergic receptor α -2B, adrenergic receptor α -2C, adrenergic receptor β -1, adrenergic receptor β -1, adrenergic
25 receptor β -3, amyloid protein precursor, angiotensin II receptor type 1, antidiuretic hormone receptor, bradykinin receptor, cannabinoid receptor, chemokine C-C (mip-1/RANTES) receptor, cholecystokinin receptor, complement C5a (anaphylotoxin) receptor, dopamine
30 receptor D1, dopamine receptor D2, dopamine receptor D3, dopamine receptor D4, dopamine receptor D5, endothelin receptor A, endothelin receptor B, f-met-leu-phe receptor, follicle stimulating hormone

receptor, glutamate receptor (metabotropic),
 gonadotropin-releasing factor receptor, growth hormone
 releasing hormone receptor, histamine H2 receptor,
 hydroxytryptamine (serotonin) receptor 1A,
 5 hydroxytryptamine (serotonin) receptor 1B,
 hydroxytryptamine (serotonin) receptor 1C,
 hydroxytryptamine (serotonin) receptor 1D,
 hydroxytryptamine (serotonin) receptor 1E,
 hydroxytryptamine (serotonin) receptor 2, insulin-like
 10 growth factor II receptor, interleukin 8 receptor A,
 interleukin 8 receptor B, lutenizing hormone/chorionic
 gonadotropin receptor, *mas* proto-oncogene, melanocyte
 stimulating hormone receptor, muscarinic acetylcholine
 receptor m1, muscarinic acetylcholine receptor m2
 15 muscarinic acetylcholine receptor m3, muscarinic
 acetylcholine receptor m4, muscarinic acetylcholine
 receptor m5, neuropeptide Y receptor, opioid- δ
 receptor, opioid- κ receptor, oxytocin receptor,
 platelet activating factor receptor, rhodopsin
 20 receptor, somatostatin receptor 1, somatostatin
 receptor 2, somatostatin receptor 3, substance K
 (neurokinin A) receptor, substance P (NK1) receptor,
 thrombin receptor, thromboxane A2 receptor, thyroid
 stimulating hormone receptor and vasoactive intestinal
 25 peptide receptor.

31. The recombinant DNA of claim 30, wherein said
 second polypeptide means is the G α 1 protein of
Saccharomyces cerevisiae or a mutant thereof which is
 selected by activation of the *S. cerevisiae* pheromone-
 30 induced signal transduction pathway upon ligand
 binding to said first polypeptide means.

32. A yeast cell transformed with the recombinant
 DNA of claim 26.

33. A yeast cell transformed with the recombinant

DNA of claim 27.

34. A yeast cell transformed with the recombinant DNA of claim 28.

5 35. A yeast cell transformed with the recombinant DNA of claim 29.

36. A yeast cell transformed with the recombinant DNA of claim 30.

37. A membrane preparation of a yeast cell transformed with the recombinant DNA of claim 26.

10 38. A membrane preparation of a yeast cell transformed with the recombinant DNA of claim 30.

39. A method for creating a recombinant DNA molecule encoding a fusion protein having a mammalian seven-transmembrane receptor polypeptide operatively-
15 linked by its carboxy-terminus to Gpalp of *S. cerevisiae*, or a protein analogous in function to said Gpalp, whereby said fusion protein couples ligand binding by said receptor polypeptide to activation of a yeast pheromone-induced signal transduction pathway,
20 which comprises:

i) creating a DNA fragment encoding the seven-transmembrane receptor and the G α protein fused at their respective carboxy- and amino-terminal ends or creating a DNA fragment encoding the seven-
25 transmembrane receptor fused at its carboxy-terminal end to the amino terminal end of a linker peptide and a G α protein fused at its amino terminal end to the carboxy-terminal end of said linker peptide, to obtain a DNA fragment encoding
30 a fusion protein;

ii) adding to the DNA fragment encoding said

fusion protein additional nucleotides that encode additional amino acids effective for directing the fusion protein to the plasma membrane of said yeast upon expression of said fusion protein to obtain a
5 DNA fragment encoding a plasma membrane-targeted fusion protein;

iii) mutagenizing the *GPA1* domain of said DNA fragment encoding a plasma membrane-targeted fusion protein to obtain a pool of DNA fragments encoding
10 mutant membrane targeted fusion proteins;

iv) linking said DNA fragments encoding mutant plasma membrane-targeted fusion proteins to a promoter effective in said yeast for expressing said plasma membrane-targeted fusion protein from
15 said DNA fragment encoding a plasma membrane-targeted fusion protein in said yeast to form a pool of mutant fusion protein expression constructs;

v) mutating the gene of a yeast, said gene being
20 homologous in function to the *FAR1* gene of *Saccharomyces cerevisiae*, to inactivate the protein homologous in function to Far1p of *Saccharomyces cerevisiae* in said yeast;

vi) constructing a diploid cell of said yeast,
25 wherein said diploid cell has one wild-type gene homologous in function to the *GPA1* gene of *Saccharomyces cerevisiae* and one inactivated copy of said gene homologous in function to the *GPA1* gene of *Saccharomyces cerevisiae*;

vii) transforming said diploid cell of said yeast with the pool of fusion protein expression
30 constructs of step iv);

viii) isolating a diploid cell of said yeast which expresses a mutant fusion protein as a part
35 of its plasma membrane;

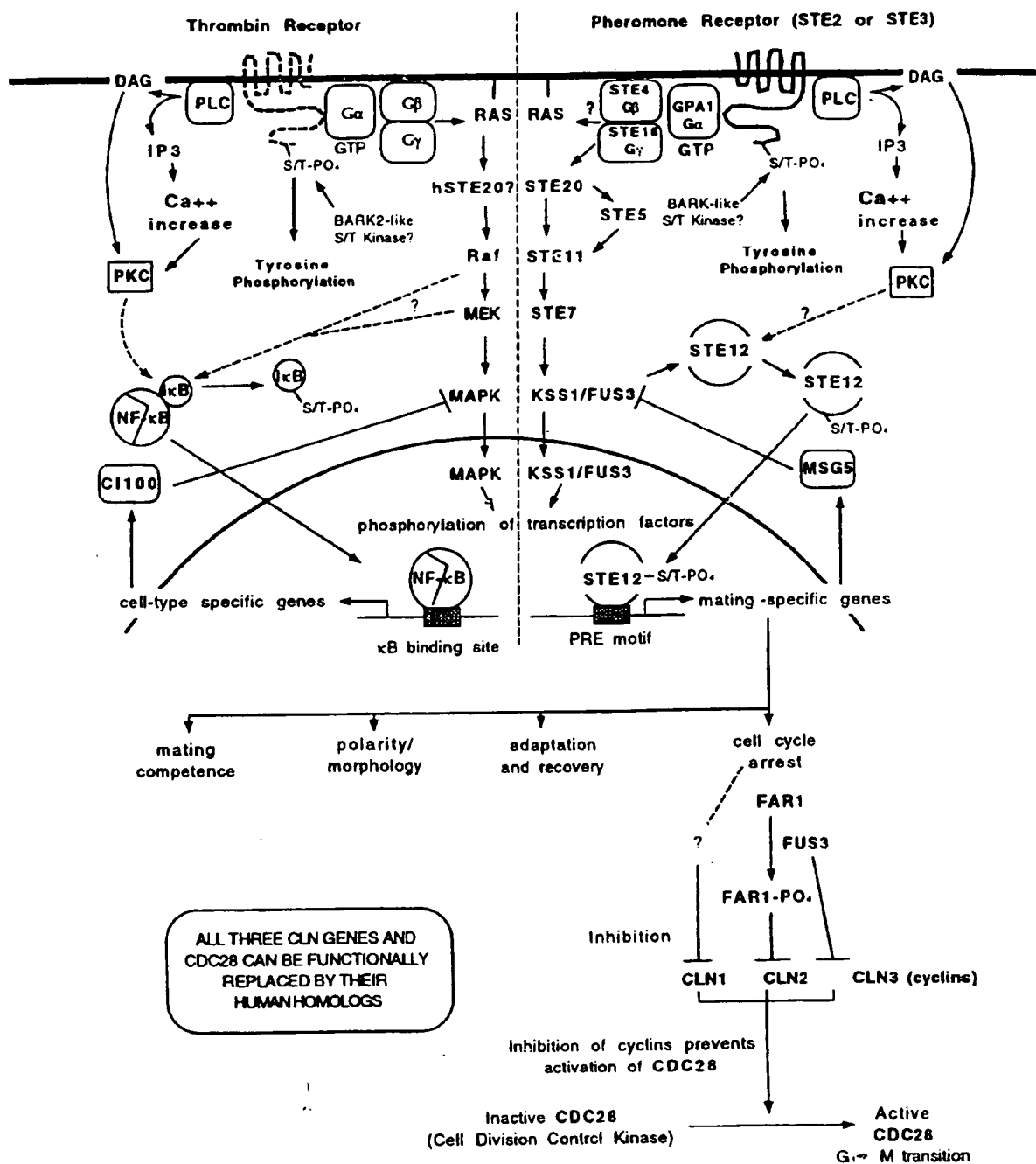
ix) transforming said diploid cell of said yeast of step viii) with a vector for expressing a marker

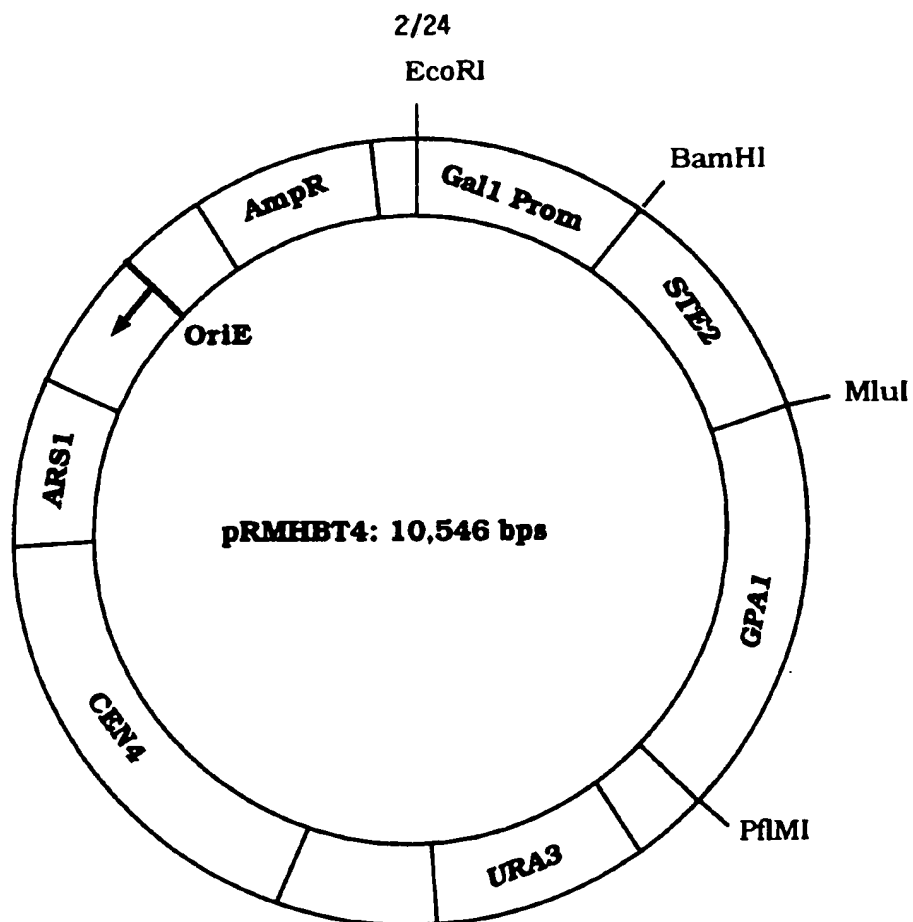
5 gene under control of a promoter homologous in function to the promoter of the *FUS1* gene of *S. cerevisiae*, thereby obtaining a diploid cell of said yeast which will grow in a medium selective for the marker gene only when the pheromone-induced signal transduction pathway of said yeast is activated;

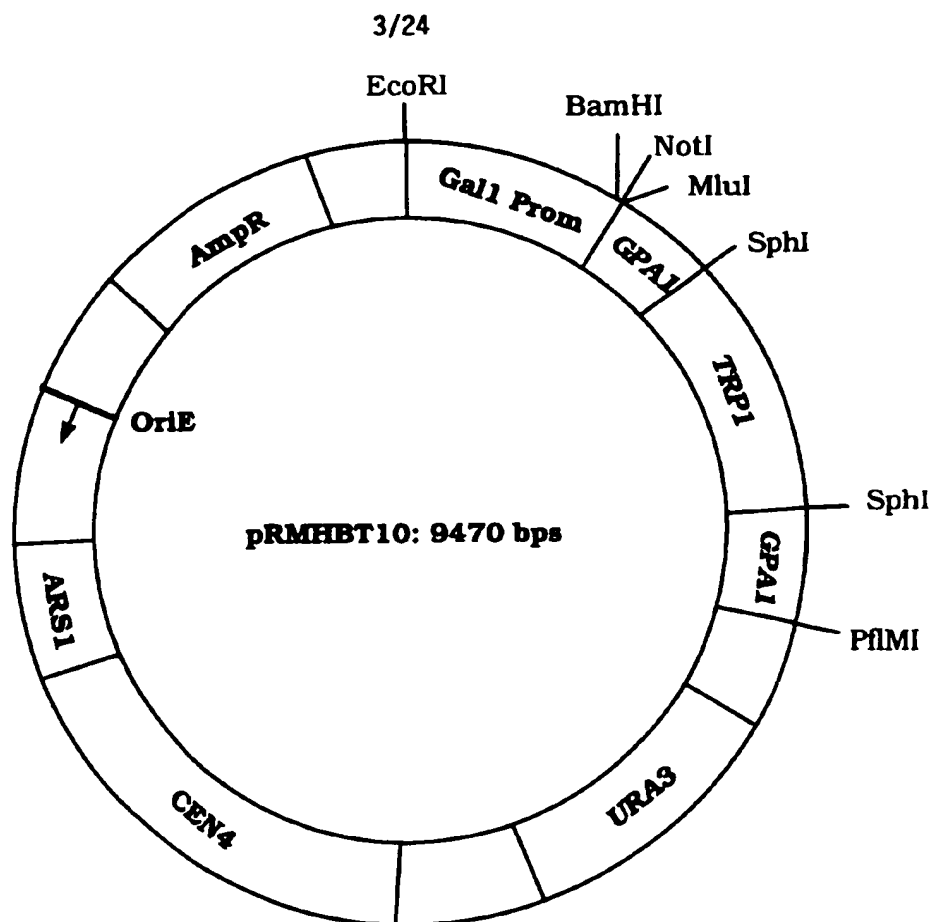
10 x) sporulating the diploid cells of step ix) to identify a haploid cell that is *gpa1*, *far1* genotype and harboring the reporter gene construct;

xi) selecting a haploid cell of said yeast by culturing the transformants of step ix) in a medium selective for the marker gene, wherein said medium also contains the ligand for said receptor; and

15 xii) cloning from said haploid cell of step xi) the DNA fragment encoding the mutant fusion protein.

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FIGURE 1HETEROTRIMERIC G-PROTEIN SIGNALING IN YEAST AND MAMMALS

**FIGURE 2**

**FIGURE 3**

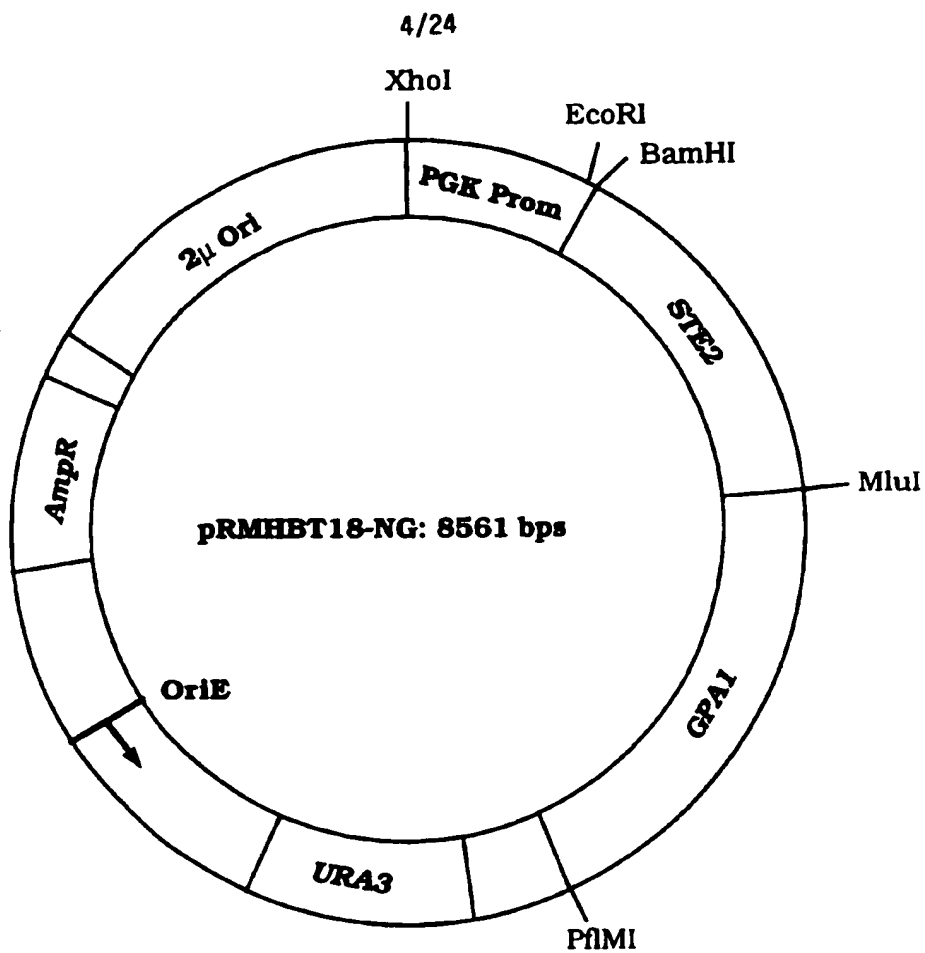
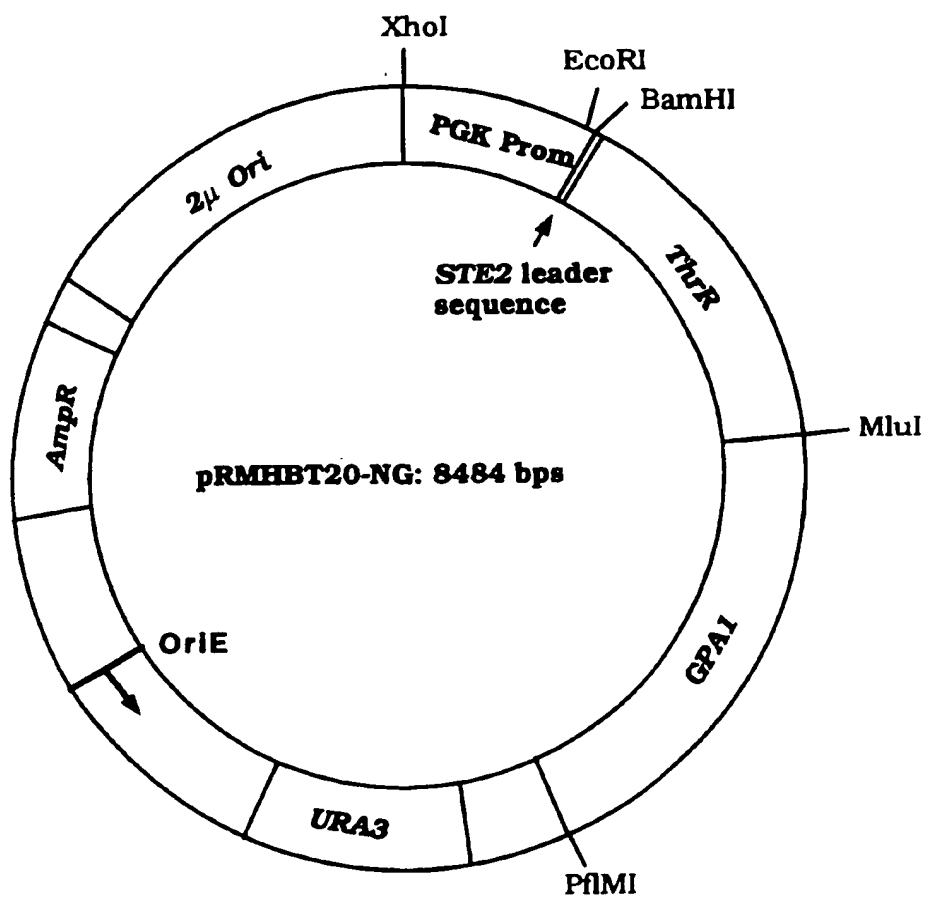


FIGURE 4

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**FIGURE 5**

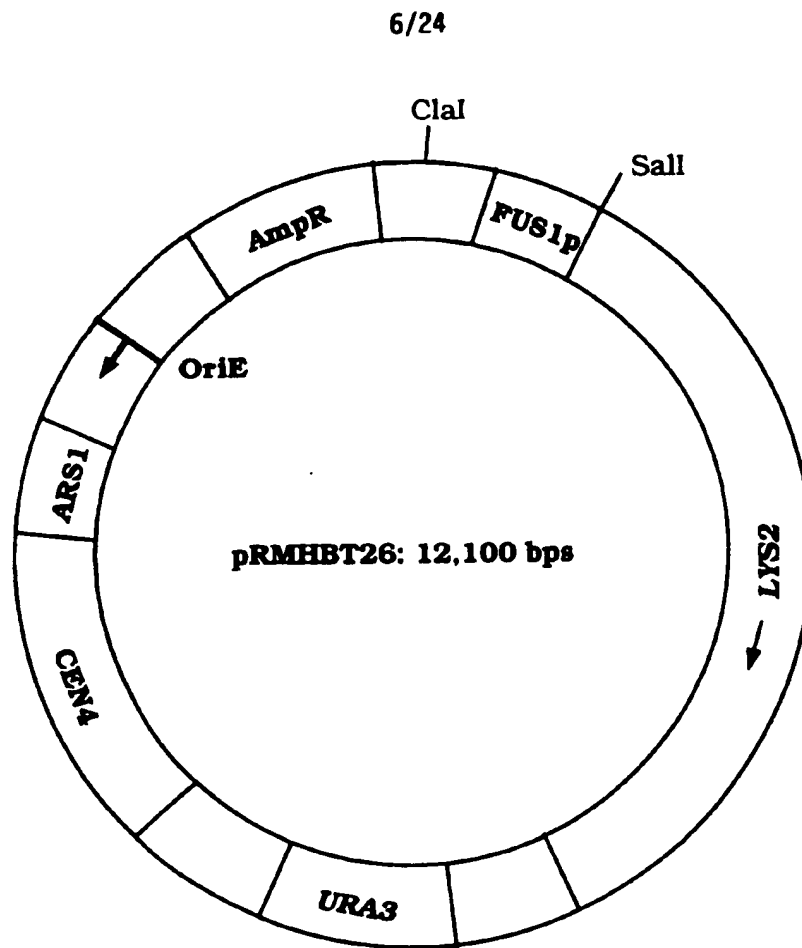


FIGURE 6

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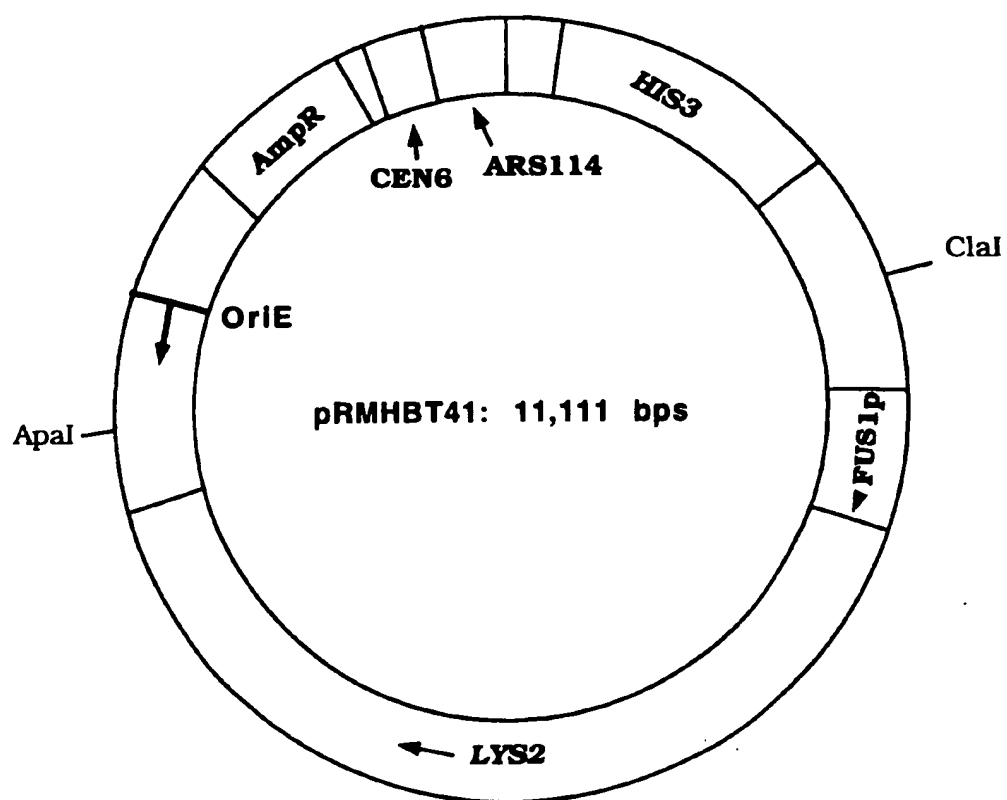


FIGURE 7

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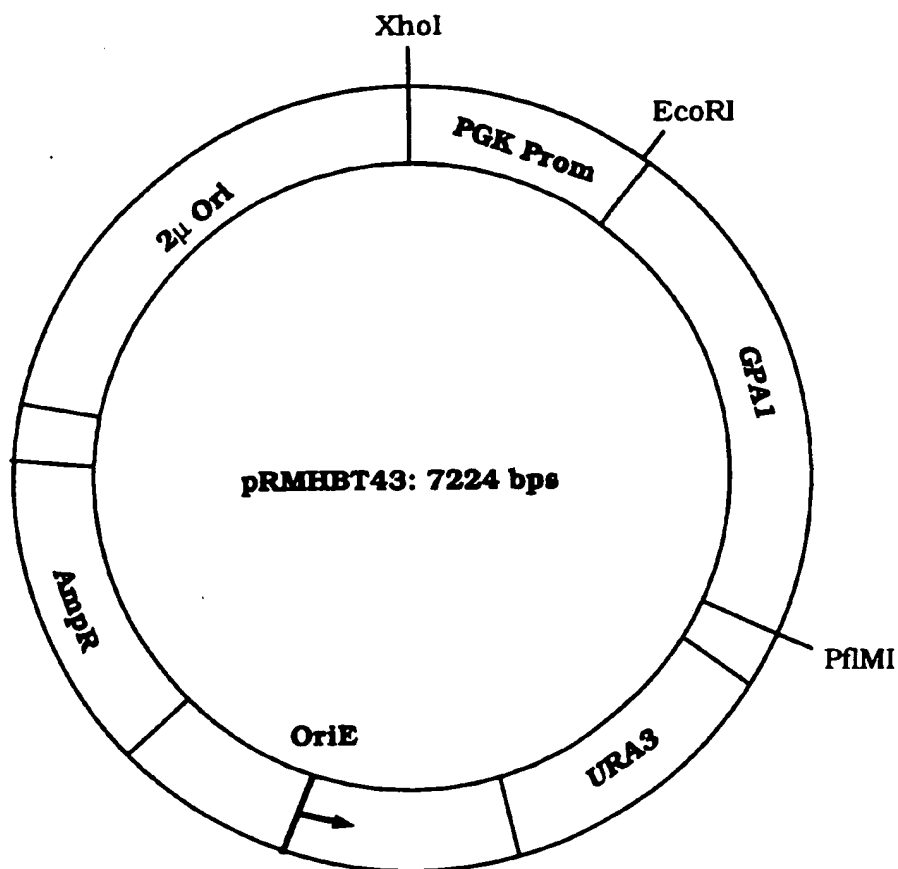
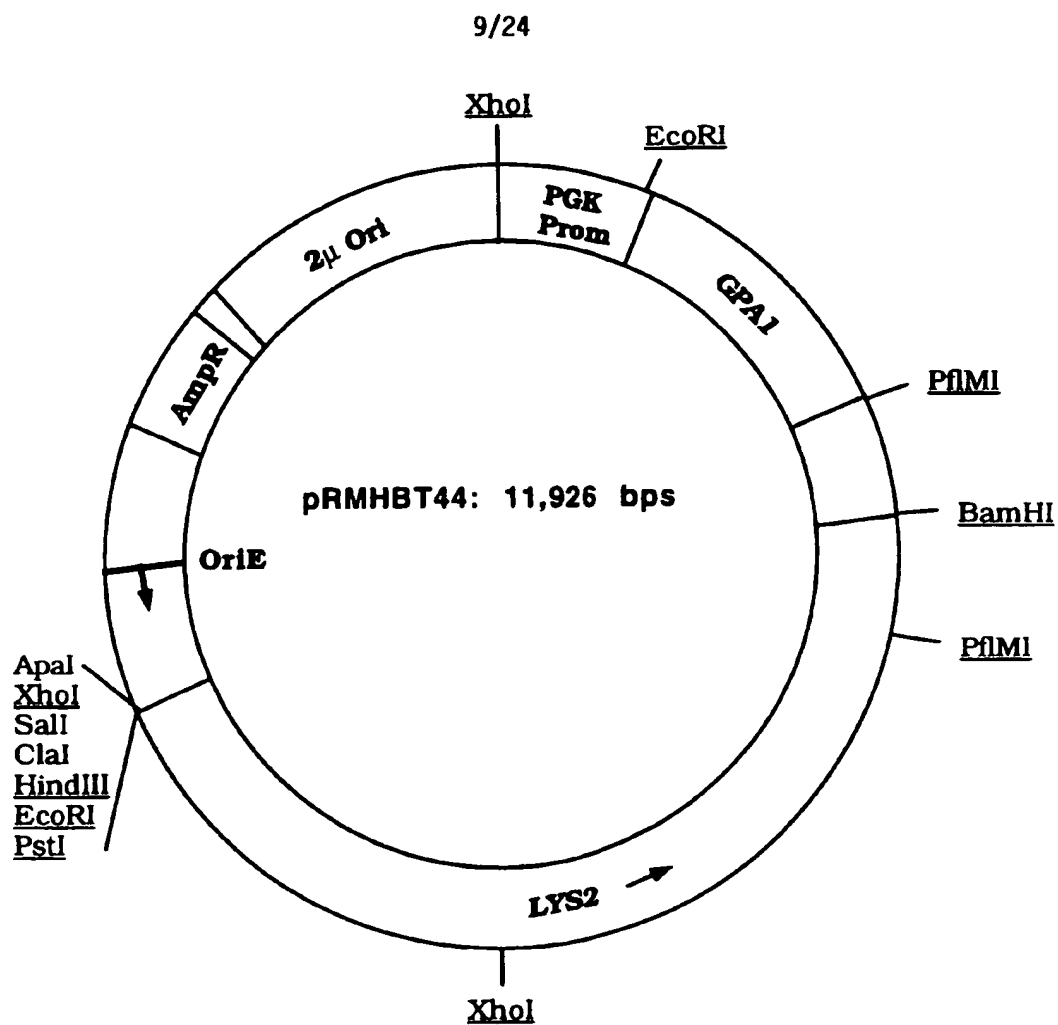


FIGURE 8

**FIGURE 9**

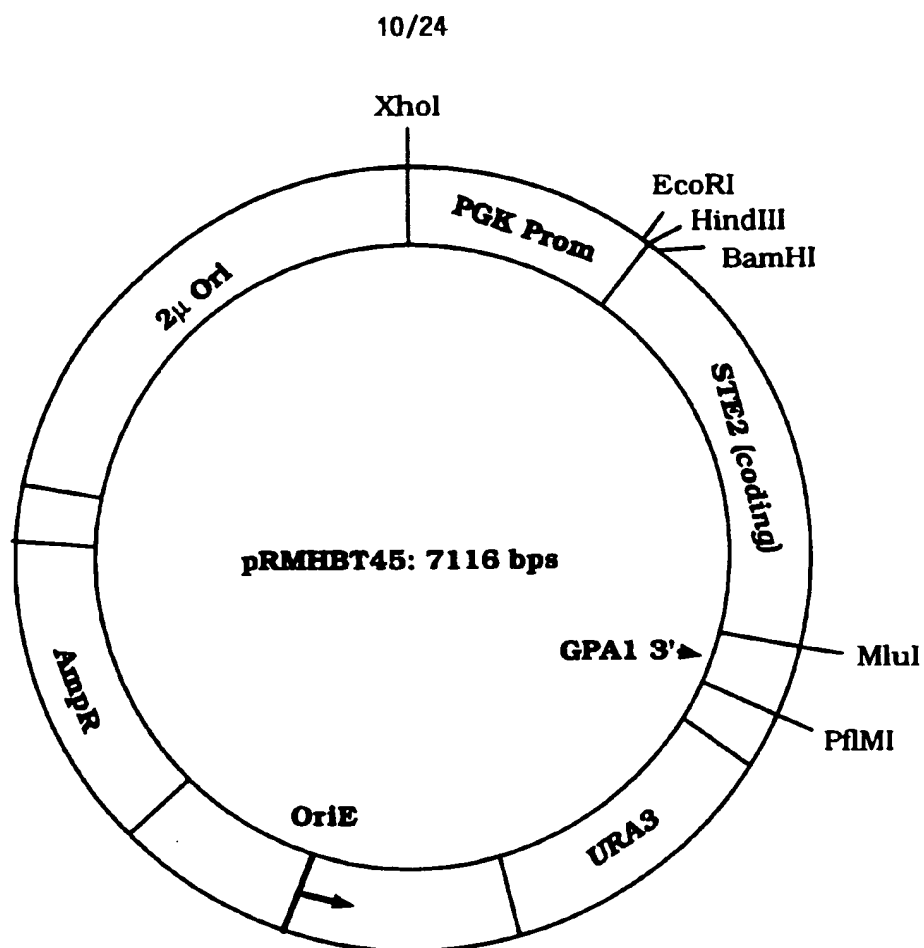


FIGURE 10

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STE2 pos. 520

1
 ATCCAAGAATCAAAAATGTCT
 +-----+-----+ 540
 TAGGTTCTTAGTTTTTACAGA

M S -

GATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGC
 -----+-----+-----+-----+-----+-----+
 CTACGCCGAGGAAGTAACTCGTTAGATAAAATACTAGGTTGCATATTAGGACCAGTTTCG

 D A A P S L S N L F Y D P T Y N P G Q S -
 ACCATTAAC TACACTTCCATATATGGGAATGGATCTACCATCACTTTTCGATGAGTTGCAA
 601 -----+-----+-----+-----+-----+-----+ 660
 TGGTAATTGATGTGAAGGTATATACCCTTACCTAGATGGTAGTGAAAGCTACTCAACGTT

 T I N Y T S I Y G N G S T I T F D E L Q -

 GGTTTAGTTAACAGTACTGTTACTCAGGCCATTATGTTTGGTGTGAGATGTGGTGCAGCT
 661 -----+-----+-----+-----+-----+-----+ 720
 CCAAATCAATTGTCATGACAATGAGTCCGGTAATACAAACCACAGTCTACACCACGTCGA

 G L V N S T V T Q A I M F G V R C G A A -

 GCTTTGACTTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAACGCCGATTTTCATT
 721 -----+-----+-----+-----+-----+-----+ 780
 CGAAACTGAACTAACAGTACACCTACTGTAGCTCTTCGTCTTTTTCGGGCTAAAAGTAA

 A L T L I V M W M T S R S R K T P I F I -

 ATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTA
 781 -----+-----+-----+-----+-----+-----+ 840
 TAGTTGGTTCAAAGTAACAAAAATTAGTAAACGTAAGACGTGAGATAAAATTTATAAAT

 I N Q V S L F L I I L H S A L Y F K Y L -

FIGURE 11A

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CTGTCTAATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCTCAGTTCATCAGTAGA
841 -----+-----+-----+-----+-----+ 900
GACAGATTAATGAGAAGTCACTGAATGCGAGAGTGGCCTAAAGGAGTCAAGTAGTCATCT
L S N Y S S V T Y A L T G F P Q F I S R -
GGTGACGTTTCATGTTTATGGTGCTACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATT
901 -----+-----+-----+-----+-----+ 960
CCACTGCAAGTACAAATACCACGATGTTTATATTAAGTTCAGGAAGAACACCGAAGATAA
G D V H V Y G A T N I I Q V L L V A S I -
GAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGGCGACAACCTTCAAAAGGATA
961 -----+-----+-----+-----+-----+ 1020
CTCTGAAGTGACCACAAAGTCTATTTTCAATAAAAGTGTCCGCTGTTGAAGTTTTCCTAT
E T S L V F Q I K V I F T G D N F K R I -
GGTTTGATGCTGACGTCGATATCTTTCACCTTTAGGGATTGCTACAGTTACCATGTATTTT
1021 -----+-----+-----+-----+-----+ 1080
CCAAACTACGACTGCAGCTATAGAAAGTGAAATCCCTAACGATGTCAATGGTACATAAAA
G L M L T S I S F T L G I A T V T M Y F -
GTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAA
1081 -----+-----+-----+-----+-----+ 1140
CATTCGCGACAATTTCCATACTAACACTGAATATTACTACAATCACGGTGGGTTCTATTT
V S A V K G M I V T Y N D V S A T Q D K -
TACTTCAATGCATCCACAATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTG
1141 -----+-----+-----+-----+-----+ 1200
ATGAAGTTACGTAGGTGTTAAAATGAACGTAGGAGTTATTTGAAATACAGTAAACAGGAC
Y F N A S T I L L A S S I N F M S F V L -
GTAGTTAAATTGATTTTATAGCTATTAGATCAAGAAGATTCCTTGGTCTCAAGCAGTTTCGAT
1201 -----+-----+-----+-----+-----+ 1260
CATCAATTTAACTAAAATCGATAATCTAGTTCTTCTAAGGAACCAGAGTTCGTCAAGCTA
V V K L I L A I R S R R F L G L K Q F D -

FIGURE 11B

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AGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATAATATTC
1261 -----+-----+-----+-----+-----+ 1320
TCAAAGGTATAAAATGAGTATTACAGTACAGTTAGAAACAACCAAGGTAGCTATTATAAG
S F H I L L I M S C Q S L L V P S I I F -
ATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACA
1321 -----+-----+-----+-----+-----+ 1380
TAGGAGCGTATGTCAAACCTTTGGTTTGGTCCCTTGTCTACAGAACTGATGACAACGTTGT
I L A Y S L K P N Q G T D V L T T V A T -
TTACTTGCTGTATTGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCA
1381 -----+-----+-----+-----+-----+ 1440
AATGAACGACATAACAGAAATGGTAATAGTAGTTACACCCGGTGCCGACGATTATTACGT
L L A V L S L P L S S M W A T A A N N A -
TCCAAAACAAACACAATTACTTCAGACTTTACAACATCCACAGATAGGTTTTATCCAGGC
1441 -----+-----+-----+-----+-----+ 1500
AGGTTTTGTTTGTGTTAATGAAGTCTGAAATGTTGTAGGTGTCTATCCAAAATAGGTCCG
S K T N T I T S D F T T S T D R F Y P G -
ACGCTGTCTAGCTTTCAAACCTGATAGTATCAACAACGATGCTAAAAGCAGTCTCAGAAGT
1501 -----+-----+-----+-----+-----+ 1560
TGCGACAGATCGAAAGTTTGACTATCATAGTTGTTGCTACGATTTTCGTCTAGAGTCTTCA
T L S S F Q T D S I N N D A K S S L R S -
AGATTATATGACCTATATCCTAGAAGGAAGGAAACAACATCGGATAAACATTTCGGAAAGA
1561 -----+-----+-----+-----+-----+ 1620
TCTAATATACTGGATATAGGATCTTCCTTCCTTTGTTGTAGCCTATTGTAAGCCTTTCT
R L Y D L Y P R R K E T T S D K H S E R -
ACTTTTGTCTTGAGACTGCAGATGATATAGAGAAAAATCAGTTTTATCAGTTGCCACACA
1621 -----+-----+-----+-----+-----+ 1680
TGAAAACAAAGACTCTGACGTCTACTATATCTCTTTTGTAGTCAAAATAGTCAACGGGTGT
T F V S E T A D D I E K N Q F Y Q L P T -

FIGURE 11C

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1681 CCTACGAGTTCAAAAAATACTAGGATAGGACCGTTTGCTGATGCAAGTTACAAAGAGGGA
-----+-----+-----+-----+-----+-----+ 1740
GGATGCTCAAGTTTTTTTATGATCCTATCCTGGCAAACGACTACGTTCAATGTTTCTCCCT

P T S S K N T R I G P F A D A S Y K E G -

1741 GAAGTTGAACCCGTCGACATGTACACTCCCGATACGGCAGCTGATGAGGAAGCCAGAAAG
-----+-----+-----+-----+-----+-----+ 1800
CTTCAACTTGGGCAGCTGTACATGTGAGGGCTATGCCGTCGACTACTCCTTCGGTCTTTC

E V E P V D M Y T P D T A A D E E A R K -

1801 TTCTGGACTGAAGATAATAATAATTTA
-----+-----+-----+-----+-----+
AAGACCTGACTTCTATTATTATTAAAT

F W T E D N N N L

1827 in STE2
1
ACGCGTGTA
-----
TGCGCACAT

T R V

202 from GPA1
1
ATGGGGTGTACAGTGAGTACGCAAACAATAGGAGACGAA
-----+-----+-----+-----+-----+
TACCCACATGTCACTCATGCGTTTGTTATCCTCTGCTT

M G C T V S T Q T I G D E -

AGTGATCCTTTTCTACAGAACAAAAGAGCCAATGATGTCATCGAGCAATCGTTGCAGCTG
-----+-----+-----+-----+-----+-----+
TCACTAGGAAAAGATGTCTTGTTTTCTCGGTTACTACAGTAGCTCGTTAGCAACGTCGAC

S D P F L Q N K R A N D V I E Q S L Q L -

GAGAAACAACGTGACAAGAATGAAATAAACTGTTACTATTAGGTGCCGGTGAGTCAGGT
-----+-----+-----+-----+-----+-----+
CTCTTGTTGCACTGTTCTTACTTTATTTTGACAATGATAATCCACGGCCACTCAGTCCA

E K Q R D K N E I K L L L L G A G E S G -

AAATCAACGGTTTTTAAACAATTAAAATTATTACATCAAGGCGGTTTCTCCCATCAAGAA
-----+-----+-----+-----+-----+-----+
TTTAGTTGCCAAAATTTTGTTAATTTTAATAATGTAGTTCCGCCAAAGAGGGTAGTTCTT

K S T V L K Q L K L L H Q G G F S H Q E -

AGGTTACAGTATGCTCAAGTGATATGGGCAGATGCCATACAATCAATGAAAATTTTGATT
-----+-----+-----+-----+-----+-----+
TCCAATGTCATACGAGTTCCTATACCCGTTCTACGGTATGTTAGTTACTTTTAAACTAA

R L Q Y A Q V I W A D A I Q S M K I L I -

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FIGURE 11D

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ATTCAGGCCAGAAACTAGGTATTCAACTTGACTGTGATGATCCGATCAACAATAAAGAT
-----+-----+-----+-----+-----+
TAAGTCCGGTCTTTTGATCCATAAGTTGAACTGACACTACTAGGCTAGTTGTTATTTCTA
I Q A R K L G I Q L D C D D P I N N K D -

TTGTTTGCATGCAAGAGAATACTGCTAAAGGCTAAAGCTTTAGATTATATCAACGCCAGT
-----+-----+-----+-----+-----+
AACAAACGTACGTTCTCTTATGACGATTTCCGATTTGAAATCTAATATAGTTGCGGTCA
L F A C K R I L L K A K A L D Y I N A S -

GTTGCCGGTGGTTCTGATTTTCTAAATGATTATGTACTGAAGTACTCAGAAAGGTATGAA
-----+-----+-----+-----+-----+
CAACGGCCACCAAGACTAAAAGATTTACTAATACATGACTTCATGAGTCTTTCCATACTT
V A G G S D F L N D Y V L K Y S E R Y E -

ACTAGGAGGCGTGTTTCAGAGTACCGGACGAGCAAAAGCTGCTTTCGATGAAGACGGAAAT
-----+-----+-----+-----+-----+
TGATCCTCCGCACAAGTCTCATGGCCTGCTCGTTTTTCGACGAAAGCTACTTCTGCCTTTA
T R R R V Q S T G R A K A A F D E D G N -

ATTTCTAATGTCAAAAGTGACACTGACAGAGATGCTGAAACGGTGACGCAAAATGAGGAT
-----+-----+-----+-----+-----+
TAAAGATTACAGTTTTTCACTGTGACTGTCTCTACGACTTTGCCACTGCGTTTTACTCCTA
I S N V K S D T D R D A E T V T Q N E D -

GCTGATAGAAACAACAGTAGTAGAATTAACCTACAGGATATTTGCAAGGACTTGAACCAA
-----+-----+-----+-----+-----+
CGACTATCTTTGTTGTCATCATCTTAATTGGATGTCCTATAAACGTTCTGAACTTGTT
A D R N N S S R I N L Q D I C K D L N Q -

GAAGGCGATGACCAGATGTTTGTAGAAAAACATCAAGGGAAATTCAAGGACAAAATAGA
-----+-----+-----+-----+-----+
CTTCCGCTACTGGTCTACAAACAATCTTTTTGTAGTTCCCTTTAAGTTCCTGTTTTATCT
E G D D Q M F V R K T S R E I Q G Q N R -

CGAAATCTTATTACGAAGACATTGCTAAGGCAATAAAGCAACTTTGGAATAACGACAAA
-----+-----+-----+-----+-----+
GCTTTAGAATAAGTGCTTCTGTAACGATTCCGTTATTTTCGTTGAAACCTTATTGCTGTTT
R N L I H E D I A K A I K Q L W N N D K -

FIGURE 11E

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GGTATAAAGCAGTGTTTTGCACGTTCTAATGAGTTTCAATTGGAGGGCTCAGCTGCATAC
-----+-----+-----+-----+-----+-----+
CCATATTTTCGTCAAAAACGTGCAAGATTACTCAAAGTTAACCTCCCGAGTCGACGTATG
G I K Q C F A R S N E F Q L E G S A A Y -

TACTTTGATAACATTGAGAAATTTGCTAGTCCGAATTATGTCTGTACGGATGAAGACATT
-----+-----+-----+-----+-----+-----+
ATGAAACTATTGTAACCTCTTTAAACGATCAGGCTTAATACAGACATGCCTACTTCTGTAA
Y F D N I E K F A S P N Y V C T D E D I -

TTGAAGGGCCGTATAAAGACTACAGGCATTACAGAAACCGAATTTAACATCGGCTCGTCC
-----+-----+-----+-----+-----+-----+
AACTTCCCGGCATATTTCTGATGTCCGTAATGTCTTTGGCTTAAATTGTAGCCGAGCAGG
L K G R I K T T G I T E T E F N I G S S -

AAATTCAAGGTTCTCGACGCTGGTGGGCAGCGTTCTGAACGTAAGAAGTGGATTTCATTGT
-----+-----+-----+-----+-----+-----+
TTTAAGTTCCAAGAGCTGCGACCACCGTCGCAAGACTTGCATTCTTCACCTAAGTAACA
K F K V L D A G G Q R S E R K K W I H C -

TTCGAAGGAATTACAGCAGTTTTATTTGTTTTAGCAATGAGTGAATACGACCAGATGTTG
-----+-----+-----+-----+-----+-----+
AAGCTTCCTTAATGTCGTCAAAATAAACAAAATCGTTACTCACTTATGCTGGTCTACAAC
F E G I T A V L F V L A M S E Y D Q M L -

TTTGAGGATGAAAGAGTGAACAGAATGCATGAATCAATAATGCTATTTGACACGTTATTG
-----+-----+-----+-----+-----+-----+
AAACTCCTACTTTCTCACTTGTCTTACGTACTTAGTTATTACGATAAACTGTGCAATAAC
F E D E R V N R M H E S I M L F D T L L -

AACTCTAAGTGGTTCAAAGATACACCGTTTATTTTGTTTTTAAATAAAATTGATTTGTTC
-----+-----+-----+-----+-----+-----+
TTGAGATTCACCAAGTTTCTATGTGGCAAATAAAACAAAATTTATTTTAACTAAACAAG
N S K W F K D T P F I L F L N K I D L F -

GAGGAAAAGGTAAAAAGCATGCCATAAGAAAGTACTTTTCTGATTACCAGGGACGTGTC
-----+-----+-----+-----+-----+-----+
CTCCTTTTCCATTTTTTCGTACGGGTATTCTTTTCATGAAAGGACTAATGGTCCCTGCACAG
E E K V K S M P I R K Y F P D Y Q G R V -
GGCGATGCAGAAGCGGTCTAAAATATTTTGAGAAGATATTTTGAGCTTGAATAAGACA
-----+-----+-----+-----+-----+-----+
CCGCTACGTCTTCGCCAGATTTTATAAACTCTTCTATAAAAACTCGAACTTATTCTGT
G D A E A G L K Y F E K I F L S L N K T -

AACAAACCAATCTACGTGAAACGAACCTGCGCTACCGATACCCAACTATGAAGTTCGTA
-----+-----+-----+-----+-----+-----+
TTGTTTGGTTAGATGCACTTTGCTTGGACGCGATGGCTATGGGTTTGATACTTCAAGCAT

FIGURE 11F

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N K P I Y V K R T C A T D T Q T M K F V -
TTGAGTGCAGTCACCGATCTAATCATCCAGCAAACCTTAAAAAAATTGGTATTATATGA
-----+-----+-----+-----+-----+
AACTCACGTCAGTGGCTAGATTAGTAGGTCGTTTTGGAATTTTTTTAACCATAATATACT
L S A V T D L I I Q Q N L K K I G I I * -

AGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAAATTCTGTTTCCGAAGATGCAAGA
-----+-----+-----+-----+-----+
TCCTTGACATATTAATTTTCATCACAAATCTATGCATTTAAGACAAAGGCTTCTACGTTCT
AGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCTTCTCCATTAGAGTCTATGATGGAA
-----+-----+-----+-----+-----+
TCCTCGTCGTCGTGGTCTTTTTTAATGATAAAAAGAAGAGGTAATCTCAGATACTACCTT
TGCCAAATGAAAAAGCCATTTTGTTCAACAGTTCTTGATCTCGTTAAATCGTTCCGGGTT
-----+-----+-----+-----+-----+
ACGGTTTACTTTTTTCGGTAAAACAAGTTGTCAAGAACTAGAGCAATTTAGCAAGGCCCAA
1851 in GPA1
1
TTCAATTGAAAAACAAGGGTAATAAAAATCGCATGAGAAAAAAAAGGTCCAG
-----+-----+-----+-----+-----+
AAGTTAACTTTTTGTTCCCATTATTTTAGCGTACTCTTTTTTTTCCAGGTC

FIGURE 11G

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535 in STE2 seq.

1
ATGTCTGATGCGGTCCCTTCATTGAGCAATCTATTTTAT
-----+-----+-----+-----
TACAGACTACGCCAGGGAAGTAACTCGTTAGATAAAATA

M S D A A P S L S N L F Y

288 in ThrR seq.

1
GCCCCGACCCGGG
---+-----+
CGGGCGTGGGCCC

A R T R A -

CCCGCAGGCCAGAATCAAAAGCAACAAATGCCACCTTAGATCCCCGGTCATTTCTTCTCA
-----+-----+-----+-----+-----+-----+
GGGCGTCCGGTCTTAGTTTTCGTTGTTTACGGTGGAATCTAGGGGCCAGTAAAGAAGAGT

R R P E S K A T N A T L D P R S F L L R -

GGAACCCCAATGATAAATATGAACCATTTTGGGAGGATGAGGAGAAAAATGAAAGTGGGT
-----+-----+-----+-----+-----+-----+
CCTTGGGGTTACTATTTTATACTTGGTAAAACCCTCCTACTCCTCTTTTTACTTTTACCCCA

N P N D K Y E P F W E D E E K N E S G L -

TAACTGAATACAGATTAGTCTCCATCAATAAAAGCAGTCCTCTTCAAAAACAACTTCCTG
-----+-----+-----+-----+-----+-----+
ATTGACTTATGTCTAATCAGAGGTAGTTATTTTCGTCAGGAGAAGTTTTTGTGGAAGGAC

T E Y R L V S I N K S S P L Q K Q L P A -

CATTCATCTCAGAAGATGCCTCCGGATATTTGACCAGCTCCTGGCTGACACTCTTTGTCC
-----+-----+-----+-----+-----+-----+
GTAAGTAGAGTCTTCTACGGAGGCCTATAAACTGGTCGAGGACCGACTGTGAGAAACAGG

F I S E D A S G Y L T S S W L T L F V P -

FIGURE 12A

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CATCTGTGTACACCGGAGTGTTTGTAGTCAGCCTCCCACTAAACATCATGGCCATCGTTG
-----+-----+-----+-----+-----+-----+
GTAGACACATGTGGCCTCACAAACATCAGTCGGAGGGTGATTGTAGTACCGGTAGCAAC
S V Y T G V F V V S L P L N I M A I V V -
TGTTTCATCCTGAAAATGAAGGTCAAGAAGCCGGCGGTGGTGTACATGCTGCACCTGGCCA
-----+-----+-----+-----+-----+-----+
ACAAGTAGGACTTTTACTTCCAGTTCTTCGGCCGCCACCACATGTACGACGTGGACCGGT
F I L K M K V K K P A V V Y M L H L A T -
CGGCAGATGTGCTGTTTGTGTCTGTGCTCCCCTTTAAGATCAGCTATTACTTTTCCGGCA
-----+-----+-----+-----+-----+-----+
GCCGTCTACACGACAAACACAGACACGAGGGGAAATTCTAGTCGATAATGAAAAGGCCGT
A D V L F V S V L P F K I S Y Y F S G S -
GTGATTGGCAGTTTGGGTCTGAATTGTGTGCTCGCTTCGTCACTGCAGCATTTTACTGTAACA
-----+-----+-----+-----+-----+-----+
CACTAACCGTCAAACCCAGACTTAACACAGCGAAGCAGTGACGTCGTAAAATGACATTGT
D W Q F G S E L C R F V T A A F Y C N M -
TGTACGCCTCTATCTTGCTCATGACAGTCATAAGCATTGACCGGTTTCTGGCTGTGGTGT
-----+-----+-----+-----+-----+-----+
ACATGCGGAGATAGAACGAGTACTGTCAGTATTCGTAAGTGGCCAAAGACCGACACCACA
Y A S I L L M T V I S I D R F L A V V Y -
ATCCCATGCAGTCCCTCTCCTGGCGTACTCTGGGAAGGGCTTCCTTCACTTGTCTGGCCA
-----+-----+-----+-----+-----+-----+
TAGGGTACGTCAAGGAGAGGACCGCATGAGACCCTTCCCGAAGGAAGTGAACAGACCGGT
P M Q S L S W R T L G R A S F T C L A I -
TCTGGGCTTTGGCCATCGCAGGGGTAGTGCCTCTCGTCCTCAAGGAGCAAACCATCCAGG
-----+-----+-----+-----+-----+-----+
AGACCCGAAACCGGTAGCGTCCCCATCACGGAGAGCAGGAGTTCCTCGTTTGGTAGGTCC
W A L A I A G V V P L V L K E Q T I Q V -

FIGURE 12B

20/24

TGCCCCGGGCTCAACATCACTACCTGTGTCATGATGTGCTCAATGAAACCCTGCTCGAAGGCT
-----+-----+-----+-----+-----+-----+
ACGGGCCCCGAGTTGTAGTGATGGACAGTACTACACGAGTTACTTTGGGACGAGCTTCCGA
P G L N I T T C H D V L N E T L L E G Y -
ACTATGCCTACTACTTCTCAGCCTTCTCTGCTGTCTTCTTTTTTGTGCCGCTGATCATTT
-----+-----+-----+-----+-----+-----+
TGATACGGATGATGAAGAGTCGGAAGAGACGACAGAAGAAAAACACGGCGACTAGTAAA
Y A Y Y F S A F S A V F F F V P L I I S -
CCACGGTCTGTTATGTGTCTATCATTGATGTCTTAGCTCTTCCGCAGTTGCCAACCGCA
-----+-----+-----+-----+-----+-----+
GGTGCCAGACAATACACAGATAGTAAGCTACAGAATCGAGAAGGCGTCAACGGTTGGCGT
T V C Y V S I I R C L S S S A V A N R S -
GCAAGAAGTCCCGGGCTTTGTTCTGTGTCAGCTGCTGTTTTCTGCATCTTCATCATTTGCT
-----+-----+-----+-----+-----+-----+
CGTTCTTCAGGGCCCGAAACAAGGACAGTCGACGACAAAAGACGTAGAAGTAGTAAACGA
K K S R A L F L S A A V F C I F I I C F -
TCGGACCCACAAACGTCCTCCTGATTGCGCATTACTCATTCCTTTCTCACACTTCCACCA
-----+-----+-----+-----+-----+-----+
AGCCTGGGTGTTTGCAGGAGGACTAACGCGTAATGAGTAAGGAAAGAGTGTGAAGGTGGT
G P T N V L L I A H Y S F L S H T S T T -
CAGAGGCTGCCTACTTTGCCTACCTCCTCTGTGTCTGTGTCAGCAGCATAAGCTCGTGCA
-----+-----+-----+-----+-----+-----+
GTCTCCGACGGATGAAACGGATGGAGGAGACACAGACACAGTCGTCTGATTTCGAGCACGT
E A A Y F A Y L L C V C V S S I S S C I -
TCGACCCCCTAATTTACTATTACGCTTCCTCTGAGTGCCAGAGGTACGTCTACAGTATCT
-----+-----+-----+-----+-----+-----+
AGCTGGGGGATTAAATGATAATGCGAAGGAGACTCACGGTCTCCATGCAGATGTCATAGA
D P L I Y Y Y A S S E C Q R Y V Y S I L -

FIGURE 12C

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TATGCTGCAAAGAAAGTTCCGATCCCAGCAGTTATAACAGCAGTGGGCAGTTGATGGCAA
 -----+-----+-----+-----+-----+-----+-----+
 ATACGACGTTTCTTTCAAGGCTAGGGTCGTCAATATTGTCGTCACCCGTCAACTACCGTT

C C K E S S D P S S Y N S S G Q L M A S -

1499 in ThrR Seq.

GTAAAATGGATACCTGCTCTAGTAACCTGAATAACAGCATATACAAAAAGCTGTTAACT
 -----+-----+-----+-----+-----+-----+-----+
 CATTTTACCTATGGACGAGATCATTGGACTTATTGTCGTATATGTTTTTCGACAATTGA
 K M D T C S S N L N N S I Y K K L L T

1827 in STE2

1
 ACGCGTGTA

 TGCGCACAT

I R V

202 from GPA1

1
 ATGGGGTGTACAGTGAGTACGCAAACAATAGGAGACGAA
 -----+-----+-----+-----+-----+-----+
 TACCCACATGTCACTCATGCGTTTGTTATCCTCTGCTT

M G C T V S T Q T I G D E -

AGTGATCCTTTTCTACAGAACAAAAGAGCCAATGATGTCATCGAGCAATCGTTGCAGCTG
 -----+-----+-----+-----+-----+-----+-----+
 TCACTAGGAAAAGATGTCTTGTTTTCTCGGTTACTACAGTAGCTCGTTAGCAACGTCGAC

S D P F L Q N K R A N D V I E Q S L Q L -

GAGAAACAACGTGACAAGAATGAAATAAACTGTTACTATTAGGTGCCGGTGAGTCAGGT
 -----+-----+-----+-----+-----+-----+-----+
 CTCTTTGTTGCACTGTTCTTACTTTATTTTGACAATGATAATCCACGGCCACTCAGTCCA

E K Q R D K N E I K L L L L G A G E S G -

AAATCAACGGTTTTTAAACAATTAAATTTATTACATCAAGGCGGTTTCTCCCATCAAGAA
 -----+-----+-----+-----+-----+-----+-----+
 TTTAGTTGCCAAAATTTTGTTAATTTTAATAATGTAGTTCCGCCAAAGAGGGTAGTTCTT

K S T V L K Q L K L L H Q G G F S H Q E -

AGGTTACAGTATGCTCAAGTGATATGGGCAGATGCCATACAATCAATGAAAATTTTGATT
 -----+-----+-----+-----+-----+-----+-----+
 TCCAATGTCATACGAGTTCACTATACCCGTCTACGGTATGTTAGTTACTTTTAAACTAA

R L Q Y A Q V I W A D A I Q S M K I L I -

ATTCAGGCCAGAAAAGCTAGGTATTCAACTTGACTGTGATGATCCGATCAACAATAAAGAT
 -----+-----+-----+-----+-----+-----+-----+
 TAAGTCCGGTCTTTTGATCCATAAGTTGAACTGACACTACTAGGCTAGTTGTTATTTCTA

FIGURE 12D

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I Q A R K L G I Q L D C D D P I N N K D -
TTGTTTGCATGCAAGAGAATACTGCTAAAGGCTAAAGCTTTAGATTATATCAACGCCAGT
-----+-----+-----+-----+-----+
AACAAACGTACGTTCTCTTATGACGATTTCCGATTTGAAATCTAATATAGTTGCGGTCA
L F A C K R I L L K A K A L D Y I N A S -
GTTGCCGGTGGTTCTGATTTTCTAAATGATTATGTACTGAAGTACTCAGAAAGGTATGAA
-----+-----+-----+-----+-----+
CAACGCCACCAAGACTAAAAGATTTACTAATACATGACTTCATGAGTCTTCCATACTT
V A G G S D F L N D Y V L K Y S E R Y E -
ACTAGGAGGCGTGTTCAGAGTACCGGACGAGCAAAAGCTGCTTTTCGATGAAGACGGAAAT
-----+-----+-----+-----+-----+
TGATCCTCCGCACAAGTCTCATGGCCTGCTCGTTTTTCGACGAAAGCTACTTCTGCCTTTA
T R R R V Q S T G R A K A A F D E D G N -
ATTTCTAATGTCAAAAGTGACACTGACAGAGATGCTGAAACGGTGACGCAAAATGAGGAT
-----+-----+-----+-----+-----+
TAAAGATTACAGTTTTCTACTGTGACTGTCTCTACGACTTTGCCACTGCGTTTTACTCCTA
I S N V K S D T D R D A E T V T Q N E D -
GCTGATAGAAACAACAGTAGTAGAATTAACCTACAGGATATTTGCAAGGACTTGAACCAA
-----+-----+-----+-----+-----+
CGACTATCTTTGTTGTATCATCTTAATTGGATGTCCTATAAACGTTTCTGAACCTTGTT
A D R N N S S R I N L Q D I C K D L N Q -
GAAGGCGATGACCAGATGTTTGTAGAAAAACATCAAGGGAAATTCAAGGACAAAATAGA
-----+-----+-----+-----+-----+
CTTCCGCTACTGGTCTACAAACAATCTTTTGTAGTTCCCTTTAAGTTCCTGTTTTATCT
E G D D Q M F V R K T S R E I Q G Q N R -
CGAAATCTTATTCACGAAGACATTGCTAAGGCAATAAAGCAACTTTGGAATAACGACAAA
-----+-----+-----+-----+-----+
GCTTTAGAATAAGTGCTTCTGTAACGATTCCGTTATTTTCGTTGAAACCTTATTGCTGTTT
R N L I H E D I A K A I K Q L W N N D K -
GGTATAAAGCAGTGTTTTGCACGTTCTAATGAGTTTCAATTGGAGGGCTCAGCTGCATAC
-----+-----+-----+-----+-----+
CCATATTTTCGTACAAAACGTGCAAGATTACTCAAAGTTAACCTCCCGAGTCGACGTATG
G I K Q C F A R S N E F Q L E G S A A Y -
TACTTTGATAACATTGAGAAATTTGCTAGTCCGAATTATGTCTGTACGGATGAAGACATT
-----+-----+-----+-----+-----+
ATGAAACTATTGTAACCTTTTAAACGATCAGGCTTAATACAGACATGCCTACTTCTGTAA
Y F D N I E K F A S P N Y V C T D E D I -

FIGURE 12E

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TTGAAGGGCCGTATAAAGACTACAGGCATTACAGAAACCGAATTTAACATCGGCTCGTCC
-----+-----+-----+-----+-----+-----+
AACTTCCCGGCATATTTCTGATGTCCGTAATGTCTTTGGCTTAAATTGTAGCCGAGCAGG
L K G R I K T T G I T E T E F N I G S S -
AAATTCAAGGTTCTCGACGCTGGTGGGCAGCGTTCTGAACGTAAGAAGTGGATTCAATTGT
-----+-----+-----+-----+-----+-----+
TTTAAGTTCCAAGAGCTGCGACCACCCGTCGCAAGACTTGCATTCTTCACCTAAGTAACA
K F K V L D A G G Q R S E R K K W I H C -
TTCGAAGGAATTACAGCAGTTTTATTTGTTTTAGCAATGAGTGAATACGACCAGATGTTG
-----+-----+-----+-----+-----+-----+
AAGCTTCCTTAATGTCTGTCAAAATAAACAAAATCGTTACTCACTTATGCTGGTCTACAAC
F E G I T A V L F V L A M S E Y D Q M L -
TTTGAGGATGAAAGAGTGAACAGAATGCATGAATCAATAATGCTATTTGACACGTTATTG
-----+-----+-----+-----+-----+-----+
AAACTCCTACTTTCTCACTTGTCTTACGTACTTAGTTATTACGATAAACTGTGCAATAAC
F E D E R V N R M H E S I M L F D T L L -
AACTCTAAGTGGTTCAAAGATACACCGTTTATTTTGTTTTTAAATAAAATTGATTTGTTC
-----+-----+-----+-----+-----+-----+
TTGAGATTACCAAGTTTCTATGTGGCAAATAAAACAAAATTTATTTTAACTAAACAAG
N S K W F K D T P F I L F L N K I D L F -
GAGGAAAAGGTAAAAAGCATGCCCATAAGAAAGTACTTTTCTGATTACCAGGGACGTGTC
-----+-----+-----+-----+-----+-----+
CTCCTTTTCCATTTTTCGTACGGGTATTCTTTTCATGAAAGGACTAATGGTCCCTGCACAG
E E K V K S M P I R K Y F P D Y Q G R V -
GGCGATGCAGAAAGCGGGTCTAAAATATTTTGAGAAGATATTTTGAGCTTGAATAAGACA
-----+-----+-----+-----+-----+-----+
CCGCTACGTCTTCGCCAGATTTTATAAACTCTTCTATAAAAACTCGAACTTATTCTGT
G D A E A G L K Y F E K I F L S L N K T -

AACAAACCAATCTACGTGAAACGAACCTGCGCTACCGATACCCAAACTATGAAGTTCGTA
-----+-----+-----+-----+-----+-----+
TTGTTTGGTTAGATGCACTTTTGCTTGGACGCGATGGCTATGGGTTTGATACTTCAAGCAT
N K P I Y V K R T C A T D T Q T M K F V -
TTGAGTGCAGTCACCGATCTAATCATCCAGCAAAACCTTAAAAAAATTGGTATTATATGA
-----+-----+-----+-----+-----+-----+
AACTCACGTCAGTGGCTAGATTAGTAGGTCGTTTTGGAATTTTTTAAACCATAATATACT
L S A V T D L I I Q Q N L K K I G I I * -

AGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAAATTCTGTTTCCGAAGATGCAAGA
-----+-----+-----+-----+-----+-----+
TCCTTGACATATTAATTTTCATCAGAAATCTATGCATTAAAGACAAAGGCTTCTACGTTCT

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FIGURE 12F

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AGGAGCAGCAGCACCAGAAAAAATTACTATTTTCTTCTCCATTAGAGTCTATGATGGAA
-----+-----+-----+-----+-----+-----+-----+
TCCTCGTCGTCGTGGTCTTTTTTAATGATAAAAAGAAGAGGTAATCTCAGATACTACCTT

TGCCAAATGAAAAAGCCATTTTGTTC AACAGTTCTTGATCTCGTTAAATCGTTCCGGGTT
-----+-----+-----+-----+-----+-----+-----+
ACGGTTTACTTTTTTCGGTAAAACAAGTTGTCAAGAACTAGAGCAATTTAGCAAGGCCCAA
1851 in GPA1
1
TTCAATTGAAAAACAAGGGTAATAAAATCGCATGAGAAAAAAAAGGTCCAG
-----+-----+-----+-----+-----+-----+-----+
AAGTTAACTTTTTGTTCCTTATTTTAGCGTACTCTTTTTTTTCCAGGTC

FIGURE 12G

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/15203

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 435/ 7.31, 29, 69.7, 91.1, 254.2, 254.21; 536/23.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 7.31, 29, 69.7, 91.1, 254.2, 254.21; 536/23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, HCAPLUS, MEDLINE, BIOSIS, WPIDS

search terms: seven transmembrane receptors, G-protein, yeast, GPA1, chimeric, fusion, hybrid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BERTIN et al. Cellular signaling by an agonist-activated receptor/G _α fusion protein. Proc. Natl. Acad. Sci. USA. September 1994, Vol. 91, pages 8827-8831, see entire document.	1-39
Y	WO 92/05244 A1 (DUKE UNIVERSITY) 02 April 1992 (02.04.92), see entire document, especially pages 3-5 and 7-19.	1-39
Y	WO 94/23025 A1 (CADUS PHARMACEUTICAL, INC.) 13 October 1994 (13.10.94), see entire document, especially pages 12-14, 37-44, 54-57, and 60.	1-39



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 NOVEMBER 1996

Date of mailing of the international search report

26 NOV 1996

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15203

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/21925 A1 (AMERICAN CYANAMID COMPANY) 17 August 1995 (17.08.95), see entire document, especially page 13-25 and page 55, top paragraph.	1-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15203

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 1/16, 1/18, 1/19, 15/12, 15/31, 15/62; C12Q 1/02; G01N 33/53